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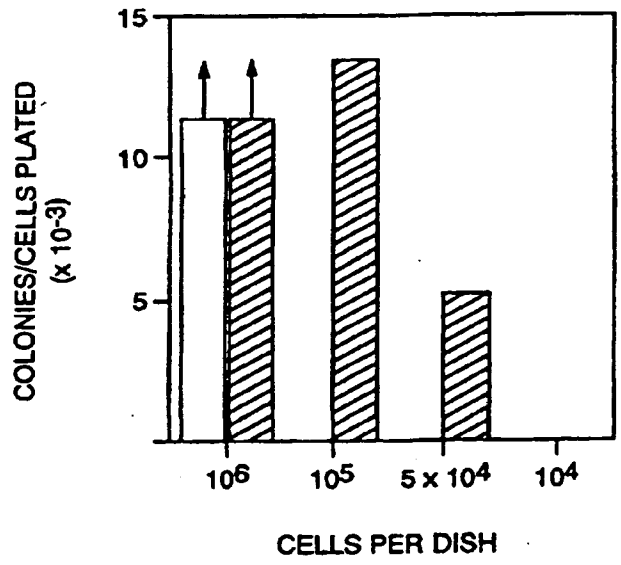

(57) Abstract

The present invention offers a developmental hematopoietic system which can be used to reproducibly and efficiently develop hematopoietic cells with a well-defined kinetic pattern. In one aspect, the method of the present invention generally includes the steps of obtaining viable stem cells from an animal donor and transfecting the cells with an expression construct including a hematopoietic gene, such as the *LH-2* gene, to produce viable hematopoietic stem cells.

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Hematopoietic Stem Cells and Methods for Generating Such Cells

Background of the Invention

The molecular mechanisms regulating the development and differentiation of the hematopoietic system (HS) are presently being elucidated. It is believed that a combination of intrinsic and extrinsic signals coordinately interact to induce self-renewal and/or differentiation of pluripotent hematopoietic stem cells (HSCs) (Huang, S. and Terstappen, L. (1992) *Nature* 360: 745-49). The differentiation of HSCs involves a series of lineage commitment steps accompanied by the acquisition of specific phenotypic characteristics. Cells gain or lose antigenic features and responsiveness to specific cytokines based on their lineage and stage of differentiation. As development proceeds, HSCs become committed to specific myeloid, lymphoid or erythroid lineages. These committed "progenitor" stem cells ultimately differentiate into a wide variety of specialized cell types which include erythrocytes, neutrophils, basophils, eosinophils, platelets, mast cells, monocytes, tissue macrophages, osteoclasts, and the T and B lymphocytes.

The first evidence of hematopoiesis in the mouse embryo is detected by the appearance of blood islands in the yolk sac at embryonic day 7.5-8. The earliest hematopoietic precursor population detected in the yolk sac is the primitive erythroid type, which consists of large nucleated cells that produce the embryonic form of hemoglobin. These transient precursors disappear after embryonic day 9. The fetal liver supersedes the yolk sac as the major hematopoietic tissue approximately at embryonic day 11, and remains the major hematopoietic organ throughout fetal life. As hematopoiesis shifts to the liver, precursors for the anucleated definite erythropoietic population and for most myeloid lineages emerge. Postnatally, hematopoiesis occurs in the bone marrow. In the adult, continuous blood cell formation is maintained by a population of primitive multipotential stem cells that have the unique capacity of self-renewal and differentiation into all different types of blood cells.

Although little is known about the genes involved in regulating hematopoiesis, transcription factors are likely to be essential regulators. Homeobox (Hox) proteins are a family of transcription factors that share a characteristic 60 amino acid domain, the homeodomain, which binds to specific DNA sequences. Hox proteins show strictly regulated patterns of expression during embryonic and fetal development, which suggest their role in the patterning of the embryo. Additional data have implicated Hox proteins in lineage-specific functions in various somatic tissues including the HS. Since the first

report on the expression of the Hox genes in hematopoietic cells, considerable attention has focused on the putative function of these genes in the HS.

The LIM domain-containing homeobox (Lhx) proteins are a recently identified class of Hox proteins. The LIM domain is a cysteine-rich amino acid sequence that was first identified in three homeobox genes *Lin*-11, *Isl*-1 and *Mec*-3. The Lhx proteins have been implicated in the control of differentiation of specific cell types. A recently cloned Lhx protein, *LH-2*, is expressed in cells in the rat fetal liver. The pattern of *LH-2* expression in the fetal liver suggests a role for *LH-2* in regulating early events in hematopoiesis, particularly when active proliferation of hematopoietic precursors is taking place.

Embryonic stem (ES) cells provide a powerful model to study the processes involved in the establishment of the hematopoietic system (HS). ES cells are totipotent cells derived from the inner mass of the developing blastocyst. These cells can be maintained in an undifferentiated state *in vitro* for extended periods in the presence of leukemia inhibitory factor (LIF). Upon LIF removal, ES cells have been shown to differentiate *in vitro* to a colony of cells known as the embryoid bodies (EBs). EBs contain a variety of cell types, including hematopoietic precursor cells. Temporal analysis of the development of these precursors indicate that they are generated in a specific ordered pattern within the EB, which is very similar to the events occurring *in vivo*. Thus, EBs initially give rise to primitive erythroid type precursors followed by the precursors of the adult erythroid lineage, as well as the myeloid multi-lineage. The similar properties of hematopoietic precursors derived from ES cells *in vitro* to their *in vivo* counterparts provide a useful model to isolate and propagate multipotential hematopoietic precursors. Additional advantages of the ES system include its accessibility to genetic manipulations and the evaluation of gene function without concern for fetal lethality.

Because of the tremendous potential of HSCs to differentiate into distinct lineages, there is great need for a continuous source of these isolated pluripotent stem cells. The transient nature and extremely low abundance of these cells *in vivo* has hampered their experimental and clinical applications. The establishment of long-term, homogeneous cultures of these cells would enhance both the investigation of the processes of lineage commitment and self-renewal, as well as the clinical strategies of bone marrow transplantation and hematopoietic cell gene therapy.

Summary of the Invention

The present invention relates to genetically modified hematopoietic stem cells derived which ectopically express a hematopoietic gene, and methods for generating such

cells. More specifically, this application relates to the ectopic expression of the LIM domain-containing homeobox protein *LH-2* in mammalian embryonic or hematopoietic stem cells. The present invention further concerns certain uses for such precursor cells, and their progeny.

One aspect of the present invention provides a method for generating renewable stem cells capable of differentiating into hematopoietic lineages, which method comprises causing ectopic expression of a hematopoietic gene, the expression of which confers a self-renewable phenotype on the stem cell. For example, the method can include a step of transfecting a stem cell with a gene expression construct encoding a hematopoietic gene, and maintaining the cells under conditions wherein the hematopoietic gene is expressed at a level conferring a self-renewable phenotype of the transfected stem cell.

In preferred embodiments, the hematopoietic gene encodes a LIM-homeobox protein, such as the *LH-2*. Exemplary LH2 genes are provided in SEQ ID Nos. 1 and 3. However, as described herein, the hematopoietic gene can be selected by such criteria as it being a gene involved in hematopoiesis characterized by a loss-of-function phenotype of a decrease in a relative number of hematopoietic cells.

In certain embodiments, the stem cell is an embryonic stem cell. In other embodiments, it will be preferred that the stem cell is a hematopoietic stem cell. Sources for hematopoietic stem cells for use in the present invention included peripheral blood, cord blood, fetal liver and bone marrow. In preferred embodiments, the stem cell is isolated from a mammal, e.g., a primate, e.g., a human. The invention also contemplates the use of stem cells from transgenic non-human mammals.

To generate the stem cell of the present invention, one preferred approach is to transfect the stem with a gene expression construct, e.g., by viral-mediated gene transfer, liposome-mediated gene transfer, or the like. The stem cell can be transfected with the gene expression construct *in vivo* (e.g., by gene therapy) or *ex vivo* (e.g., in culture).

In preferred embodiments, the subject method also includes the step of expanding the transfected stem cell by contact with Steel factor. It may also include the further step of contacting the transfected stem cell with one or more factors selected from the group consisting of erythropoietin (EPO), thrombopoietin, granulocyte/macrophage colony stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), and an interleukin. Such factors can be used to differentiate the stem cells into such hematopoietic cells as myeloid, lymphoid, and/or erythroid cells.

Another aspect of the present invention provides a substantially pure population of viable stem cells, which cells have been genetically modified to ectopically express a

hematopoietic gene, the expression of which confers a self-renewable phenotype on the stem cell. In certain embodiments, the stem cells, or the progeny thereof, can be formulated into pharmaceutical composition.

Another aspect of the present invention provides a method for treating a disorder characterized by a reduced number of hematopoietic precursors or their progeny. In general, the method includes introducing, into the subject animal, a pharmaceutical composition of the stems cells (and/or the progeny thereof) of the present invention. The instant method can be used to treat, for example, disorders resulting from bone marrow deficiency, cancer treatment involving radio- or chemotherapy, and disorders of an inherent or acquired anemia.

In another aspect of the present invention, the instant pharmaceutical preparations of cells can be used to treat a disorder characterized by an alteration in the immune system, e.g., an auto-immune disease, AIDS or the like.

Still another aspect of the present invention provides a conditioned media generated by a stem cell culture comprising stem cells which ectopically express a hematopoietic gene which confers a self-renewable phenotype on the stem cell. Similarly, the present invention also provides a purified or semi-purified preparation of one or more factors generated by a stem cell culture comprising stem cells which ectopically express a hematopoietic gene which confers a self-renewable phenotype on the stem cell.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Detailed Description of the Drawings

Figure 1 is an illustration of the pathways of differentiation of hematopoietic stem cells.

Figure 2 is a photograph depicting the typical morphology of the two types of colonies present when EBs cultured for 6 days are replated in the presence of erythropoietin (EPO) and steel factor (SF). The small tight primitive erythroid type colony (p) consists of large nucleated hemoglobinized (red) erythrocytes which express the fetal type of globins. The larger and irregularly shaped colony is a definite erythroid type of colony (d). This colony consists predominantly of anucleated hemoglobinized erythrocytes and their immediate precursors such as reticulocytes and erythroblasts.

Figures 3A and 3B are graphs depicting the frequency of erythroid precursors in EBs cultured for 6 days derived from the differentiated ES cell line CCE transduced with the Murine Stem Cell Virus (MSCV) retroviral vector (MSCV) or with this vector containing the *LH-2* gene (MSCV-*LH-2*). (3A) Frequency of precursor for the primitive erythroid lineage. (3B) Frequency of precursors for the definite erythroid lineage.

Figure 4 is a graph depicting the frequency of precursors for the primitive erythroid lineage in EBs cultured for 6 days derived from differentiated J1 ES cells.

Figure 5 is a schematic of the structure of the murine stem cell virus vector (MSCV, upper panel) and the structure of the *LH-2*- containing MSCV (MSCV-*LH-2*, lower panel). The arrows indicate transcriptional starts. *LH-2* is under the control of the modified LTR promoter. This vector contains the neomycin phosphotransferase (neo) gene under the control of the internal pgk promoter. MCS indicates the multiple cloning site. p(A) represents the polyadenylation site. Leading to that the *LH-2* gene is present on a polycistronic transcript also containing the neo gene, while the neo gene is present on two different messages. SD and SA denote splice site donor and acceptor sites, and ψ^+ denotes the extended packaging region for high viral titre. All other virus-specific genes are present in the BOSC-23 cell line.

Figure 6 is a photograph depicting the morphology of the "stem cells" in methyl cellulose. This type of colony was generated by replating EBs cultured for 6 days which were derived from CCE cells transfected with the MSCV-*LH-2* vector. The particular colony is derived from the CCE MSCV-*LH-2* subclone 7.

Figures 7 and 8 are photographs of methylcellulose colony assays described in Example 4.

Figure 9 is a graph illustrating the effect of conditioned medium on HPC colony formation.

Detailed Description of the Invention

I. Overview of Method

True hematopoietic stem cells have two unique characteristics. First, they are pluripotent; that is, they are capable of differentiating into all mature blood cell types. Second, they are self-renewing; that is, they are capable of dividing and maintaining their pluripotentiality. Hematopoietic stem cells have been characterized, including developmental potential, functional characteristics and requirements for maintenance in culture. However, while the hematopoietic stem cell cultures described in the art have a capacity for self-renewal, the capacity for such self-renewal is nevertheless substantially limited relative to the apparent renewal capacity of stem cells *in vivo*.

The present invention offers a developmental hematopoietic system which can be used to reproducibly and efficiently develop hematopoietic cells with a well-defined kinetic pattern. In one aspect, the method of the present invention generally includes the steps of obtaining viable stem cells from an animal donor and transfecting the cells with an expression construct including a hematopoietic gene, such as the *LH-2* gene, to produce viable hematopoietic stem cells. In another embodiment, an endogenous hematopoietic gene is activated by homologous recombination with a gene activation construct.

A salient feature of these cells is that they can be maintained in culture as totipotent cell lines for many generations. From the data provided in the appended examples, it is clear that this protocol can be used to generate a multilineage precursor that is capable of renewing itself and has maintained the ability to differentiate into a variety of mature hematopoietic cell types. This finding is important because the abundance of stem cells naturally is very low. Thus, a pure and homogenous population of these cells has been substantially unrealized by techniques available today.

A hematopoietic stem cell composition of the present invention is characterized by being able to be maintained in culture for extended periods of time, being capable of selection and transfer to secondary and high order cultures, and being capable of differentiating into the various hematopoeitic lineages, which may include various

lymphocytic and myelomonocytic lineages, such as B and T lymphocytes, monocytes, macrophages, neutrophils, erythrocytes and the like.

One aspect of the present invention relates to a cellular composition comprising stem cells genetically modified to express a hematopoietic gene, e.g., a transcription factor, e.g., a LIM-homeobox gene. As an illustration, *LH-2*-expressing embryonic stem cells can be used to establish cultures of primative or early hematopoietic cells, which are capable of self-renewal. Stem cells of the present invention include cells derived from human and non-human sources.

The stem cells may be grown in culture in an appropriate nutrient medium, including, but not limited to, conditioned medium, a co-culture with an appropriate stromal or other feeder layer, adhesion molecules, or a medium comprising a synthetic combination of growth factors which are sufficient to maintain the growth and (optionally) differentiation of the subject genetically modified stem cells.

The genetically modified stem cells of the invention, and the progeny thereof, can be used in a variety of applications. These include but are not limited to transplantation or implantation of the engineered cells *in vivo*; screening cytotoxic compounds, allergens, growth/regulatory factors, pharmaceutical compounds, etc., *in vitro*; elucidating the mechanism of certain diseases; studying the mechanism by which drugs and/or growth factors operate; and the production of biologically active products, to name but a few. Thus, the cells can be used therapeutically for treatment of illnesses requiring bone marrow transplantation, such as all cancer treatments involving radio- or chemotherapy, ii) a possible cure for auto immune diseases by replacing pathogenic lymphocytes iii) a possible cure for different kinds of inherited or acquired anemias, iv) AIDS if the possibility exists that the HIV infected compartment can be replaced with healthy cells by expanding a small number of the patients own non-infected stem cells.

In another aspect, a source of hematopoietic stem cells, or the progeny thereof, are provided by culturing stem cells in the presence of feeder layer cells including feeder cells which have been engineered to express *LH-2*. Premised on the notion that *LH-2* causes expression of a paracrine factor(s) involved in the self-renewal phenotype of the hematopoietic cell, this embodiment would permit the *ex vivo* expansion of wild-type stem cells, e.g., the stem cells are not themselves genetically modified. For embodiments wherein the stem cell, or a progeny thereof, are to be used therapeutically, such feeder cell layers can be preferred.

In a related manner, another aspect of the present invention makes available a conditioned media, or purified or semi-purified source of factor(s) produced by cells ectopically expressing *LH-2*. That is, any inductive factor(s) found in the supernatant of the genetically modified stem cells described herein can be provided in some form isolated

from the source cell. The present invention also contemplates the cloning of such *LH-2* induced factors, wherein the recombinant protein can be provided, e.g., in purified form, or expressed from cells in a co-culture. As above, this embodiment permits the *ex vivo* expansion of wild-type stem cells, e.g., the stem cells are not genetically modified.

Yet another aspect of the present invention concerns the use of the subject hematopoietic gene constructs for *in vivo* therapies. Thus, by gene therapy approach, the subject methods provides a means for augmenting or correcting deficiencies in the hematopoietic system, or a portion thereof, directly in a patient.

Still another aspect of the invention relates to the generation of hematopoietic stem cells which are lineage restricted. As described below, the ability of an otherwise totipotent stem cell to differentiate into any hematopoietic cell can be restricted by introducing a loss-of-function mutation to a gene critical to development of one or more particular lineages. For instance, loss-of-function mutations to GATA-3 or NFAT can be used to reduce the ability of a hematopoietic stem to differentiate to a T cell. Likewise, mutation of PU.1 or Oct2 can restrict differentiation to non-B cell lineages. Other loss-of-function mutations are described below which can be used to reduce differentiation to such lineages as, for example, monocytes, neutrophils, eosinophils, basophils, megakaryocytes and/or erthrocytes.

II. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

The term "lineage committed cell" refers to a stem cell that is no longer pluripotent but has become restricted to a specific lineage, e.g., a myeloid, lymphoid, erythroid lineage. the lineage committed cell subsequently differentiates to specialized cell types, e.g., erythrocytes, T and B lymphocytes.

The term "stem cell" refers to an undifferentiated cell which is capable of self-renewal, i.e., proliferation to give rise to more stem cells, and may give rise to lineage committed progenitors which are capable of differentiation and expansion into a specific lineage. In a preferred embodiment, the term "stem cell" refers to a generalized mother cell whose descendants (progeny) specialize, often in different directions, by differentiation, e.g., by acquiring completely individual characters, as occurs in progressive diversification of embryonic cells and tissues. As used herein, the term "stem cells" refers generally to both embryonic and hematopoietic stem cells from mammalian origin, e.g., human.

A stem cell composition is characterized by being able to be maintained in culture for extended periods of time, being capable of selection and transfer to secondary and higher order culture, and being capable of differentiating into various lymphoid or myeloid lineages, particularly B and T lymphocytes, monocytes, macrophages, neutrophils, erythrocytes and the like.

As used herein, the term "embryonic stem cell" means a pluripotent, blastocyst-derived cell that retains the developmental potential to differentiate into all somatic and germ cell lineages (for review, *see* Robertson, E. J. (1986) *Trends in Genetics* 2: 9-13). This cell type is also referred to as an "ES cell".

As used herein, the term "hematopoietic stem cell" (HSC) means a population of cells capable of both self-renewal and differentiation into all defined hematopoietic lineages, i.e., myeloid, lymphoid or erythroid lineages; and limiting number of cells are capable of repopulating the hematopoietic system of a recipient who has undergone myeloablative treatment. HSCs can ultimately differentiate into hematopoietic cells, including without limitation, common lymphoid progenitor cells, T cells (e.g., helper, cytotoxic, and suppressor cells), B cells, plasma cells, natural killer cells, common myeloid progenitor cells, monocytes, macrophages, mast cells, leukocytes, basophils, neutrophils, eosinophils, magakaryocytes, platelets, and erythroids. HSCs are identifiable by the presence of cell surface antigens of primitive phenotypes, e.g., CD34+Thy-1^{+}Lin^{-}, and negative staining for lineage-specific antigens.

The term "hematopoietic gene" refers to a gene that encodes a protein product involved in the processes of self-renewal and/or differentiation of HSCs ex vivo and in vivo. The *LH-2* gene provides an example of a hematopoietic gene since the *LH-2* gene is expressed in developing hematopoietic tissues, e.g. fetal liver, as well as certain lymphoid malignancies and lymphoid malignancies (*Oncogene* 12, 1205-66); ectopic expression of this gene in embryonic stem cells results in the enhanced production of hematopoietic precursors, e.g., precursors to the definite erythroid lineage; and homozygous mice carrying a loss-of-function mutation of the *LH-2* gene show a decrease number of definitive erythroid precursor cells and multipotential progenitor cells.

The term "hematopoietic gene construct" comprises a nucleic acid molecule, e.g., a vector, containing the subject hematopoietic gene operably linked in a manner capable of replicating and expressing the gene in a host cell. The hematopoietic gene construct can be introduced into a recipient human or non-human cell by nucleic acid-mediated gene transfer.

As used herein, "heterologous DNA" or "heterologous nucleic acid" include DNA that does not occur naturally as part of the genome in which it is present or which is found in a location or locations in the genome that differs from that in which it occurs in nature.

Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell. Generally, although not necessarily, such DNA encodes RNA and proteins that are not normally produced by the cell in which it is expressed. Heterologous DNA may also be referred to as foreign DNA. Any DNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which is expressed is herein encompassed by heterologous DNA.

The terms "homeobox gene" and "homeobox protein" refer to the gene and gene product, respectively, of a family of structurally related transcription factors that share a characteristic 60 amino acid helix-turn-helix DNA binding domain (Levine and Hoey (1988). The terms "homeobox" and "homeodomain" protein are used interchangeably herein.

As used herein, the term "*LH-2* protein" refers to a member of the Lhx subclass of homeobox proteins. These proteins contain a novel Cys-His structural motif known as a LIM domain for *lin-11 isl-1 mec-3* (Xu, Y. (1993) *Proc. Natl. Acad. Sci. USA* 90, 227-31).

The terms "protein", "polypeptide" and "peptide" are used interchangeably herein.

As used herein, the term "substantially pure", with respect to stem cells, refers to a population of stem cells that is at least about 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% pure, with respect to stem cells making up a total cell population. Recast, the term "substantially pure" refers to a population of stem cell of the present invention that contain fewer than about 20%, more preferably fewer than about 10%, most preferably fewer than about 5%, of lineage committed cells in the original unamplified and isolated population prior to subsequent culturing and amplification.

The term "culture medium" is recognized in the art, and refers generally to any substance or preparation used for the cultivation of living cells. Accordingly, a "tissue culture" refers to the maintenance or growth of tissue, e.g., explants of organ primordia or of an adult organ *in vitro* so as to preserve its architecture and function. A "cell culture" refers to a growth of cells *in vitro*; although the cells proliferate they do not organize into tissue *per se*.

The term "conditioned media" refers to the supernatant, e.g. free of the cultured cells/tissue, resulting after a period of time in contact with the cultured cells such that the media has been altered to include certain paracrine and/or autocrine factors produced by the cells and secreted into the culture.

As used herein, the term "cellular composition" refers to a preparation of cells, which preparation may include, in addition to the cells, non-cellular components such as cell culture media, e.g. proteins, amino acids, nucleic acids, nucleotides, co-enzyme, anti-

oxidants, metals and the like. Furthermore, the cellular composition can have components which do not affect the growth or viability of the cellular component, but which are used to provide the cells in a particular format, e.g., as polymeric matrix for encapsulation or a pharmaceutical preparation.

As used herein, the term "animal" refers to mammals, preferably mammals such as humans. Likewise, a "patient" or "subject" to be treated by the method of the invention can mean either a human or non-human animal.

As used herein, a "transgenic animal" is any animal, preferably a non-human mammal, bird or an amphibian, in which one or more of the cells of the animal contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or *in vitro* fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. In the typical transgenic animals described herein, the transgene causes cells to express a recombinant form a homeobox protein. However, transgenic animals in which the recombinant homeobox gene is silent are also contemplated, as for example, the FLP or CRE recombinase dependent constructs described below. Moreover, "transgenic animal" also includes those recombinant animals in which gene disruption of one or more homeobox genes is caused by human intervention, including both recombination and antisense techniques.

The "non-human animals" of the invention include vertebrates such as rodents, non-human primates, miniature swine, baboon, sheep, dog, cow and other non-human mammals. The term "chimeric animal" is used herein to refer to animals in which the recombinant gene is found, or in which the recombinant is expressed in some but not all cells of the animal. The term "tissue-specific chimeric animal" indicates that a recombinant hematopoietic gene is present and/or expressed or disrupted in some tissues but not others.

As used herein, the term "transgene" means a nucleic acid sequence (encoding, e.g., a hematopoietic protein, or pending an antisense transcript thereto), which is partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a

knockout). A transgene can include one or more transcriptional regulatory sequences and any other nucleic acid, such as introns, that may be necessary for optimal expression of a selected nucleic acid.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. The term "expression vector" includes plasmids, cosmids or phages capable of synthesizing the subject hematopoietic-protein encoded by the respective recombinant gene carried by the vector. Preferred vectors are those capable of autonomous replication and/expression of nucleic acids to which they are linked. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. Moreover, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transduction", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transduced cell expresses a recombinant form of a hematopoietic protein or, where anti-sense expression occurs from the transferred gene, the expression of a naturally-occurring form of the hematopoietic protein is disrupted.

A "disease of a hematopoietic cell" refers to any condition characterized by impairment of any normal function of a hematopoietic cell. The diseases of hematopoietic cells that can be treated utilizing the cells of the present invention include, without limitation, genetic disorders (e.g., Adenosine Deaminase Deficiency, Fanconis' Anemia, and hemoglobinopathies such as Sickle Cell Anemia, Thalassemias, and Hemoglobin C Disease), as well as diseases acquired by infectious or non-infectious means (e.g., Acquired Immune Deficiency Syndrome and leukemias).

By "gene product" it is meant a molecule that is produced as a result of transcription of a gene. Gene products include RNA molecules transcribed from a gene, as well as proteins translated from such transcripts.

III. Genes and Vectors

A. Recombinant Genes

As indicated above, the present invention provides, in one aspect, stem cells that are genetically modified to ectopically express a hematopoietic gene which enhances a self-renewal phenotype and permits expansive terminal differentiation of the stem cell into mature hematopoietic lineages. The appended examples provide an illustrative

embodiment of a stem cell engineered to recombinantly express the LIM-homeobox protein *LH-2*. However, from the description provided herein, the skilled artisan will appreciate that other embodiments of the subject cells can be practiced, including those which are generated by the use of other hematopoietic gene constructs.

As further guidance, it is noted that one criterion for selecting an appropriate hematopoietic gene for use in the present invention is based on observing the phenotype conferred by a loss-of-function mutation to a gene. For instance, as described in the appended examples, a suitable hematopoietic gene may be selected on the basis that loss-of-function mutations to that gene result in a decrease in the ability of a stem cell to differentiate into one or more mature hematopoietic cell types. For instance, loss-of-function mutations to the *LH-2* gene result in loss of mature hematopoietic cells in transgenic animals. That is, *LH-2* is critical to hematopoiesis. In opposite to the loss-of-function mutation, ectopic expression (gain-of-function) of the wild-type *LH-2* gene in the cells of the present invention, as the examples demonstrate, results in the generation of the desired stem cell phenotype. Thus, the phenotype of the loss-of-function mutation serves as a marker to identify genes which may fit the paradigm of the engineered hematopoietic cells of the present invention.

Hematopoietic genes for use in the instant invention may also (or alternatively) be selected based on an observation that the gene is expressed during hematopoietic development, particularly during the earliest stages of hematopoietic commitment. In other embodiments, the recombinant hematopoietic gene can be selected because it is overexpressed in certain forms of leukemia, and thus, may encode a protein involved in the processes of self-renewal and/or differentiation of HSCs.

One class of hematopoietic gene constructs envisioned by the present invention is derived from genes encoding transcription factors that are expressed in the hematopoietic system in a developmental or lineage-specific manner. Preferred hematopoietic genes are selected from the family of homeobox proteins. Such proteins are characterized by including a homeobox domain, e.g., helix-turn-helix motif, a primary sequence motif for specific DNA-binding (see, for example, Levine et al, (1988) *Cell* 55:537-540; Robertson, (1988) *Nature* 336:522-524, and references therein). Homeobox genes have been implicated in the patterning of the embryo, and in lineage-specific functions in various somatic tissues, including the hematopoietic system.

In preferred embodiments, the homoeobox protein is a member of the LIM subclass of homeobox proteins, e.g., a LIM-homeobox protein. These proteins contain a novel Cys-His structural motif known as a LIM domain for *lin-11 isl-1 mec-3* (Xu et al. (1993) *PNAS* 90, 227-231). Thus, a LIM-homeobox protein includes both a homeodomain and at least one LIM domain. An exemplary LIM-homeobox gene is denoted *LH-2* (see,

for example, Xu et al., *supra*; and GenBank Accession numbers U11701 [human] and L06804 [rat]). The full-length *LH-2* protein contains a homoeodomain and two LIM domains. The *LH-2* protein is expressed in developing hematopoietic tissues, e.g. fetal liver, as well as lymphoid malignancies, e.g. chronic myelogenious leukemia, lymphoid malignancies.

Additional members of this class of homeobox proteins include: the Lhx3 gene product (see, for example, Zadanov et al. (1995) *Dev Dyn* 202:354-364; and GenBank Accession number L40482 [mouse]); *isl-1*, *isl-2*, *isl-3* and the like (see, for example, Dong et al. (1991) *Mol Endocrin* 5:1633-1641; and Gong et al. (1995) *J Biol Chem* 270:3335-3345); *islet-1*, *islet-2*, and the like (see, for example, Tanizawa et al. (1994) *Diabetes* 43:935-941; and GenBank Accession numbers U07559 [human, islet-1] and L35571 [rat, islet-2]); LIM-1, LIM-2, LIM-3 and the like (see, for example, Fujii et al. (1994) *Dev Dyn* 199:73-83; Barnes et al. (1994) *Dev Biol* 161:168-178; Seidah et al. (1994) *DNA Cell Biol* 13:1163-1180; and GenBank Accession number L38249 [mouse, Lim3a]); and Lmx-1 (German et al. (1992) *Genes Dev* 6:2165-2176).

Another candidate hematopoietic gene for use in the subject invention is the c-myb gene. That gene is associated with all types of hematopoietic cells. Its expression is high in immature cells of all lineages, including lymphoid, myeloid and erythroid lineages, but is low or non-existent in mature cells. The myb proteins are implicated as promoters of growth. Although c-myb plays an apparently important role in hematopoiesis, its expression in all immature cells suggests that it does not act as a key factor in defining individual cell lineages. Rather, the expression of c-myb may define immature cells which are able to proliferate and to differentiate along individual pathways. Such a transcription factor can, provided as the hematopoietic gene in the subject method, facilitate the generation of an uncommitted hematopoietic stem cell.

Another class of transcription factors which may include members useful in the present invention include zinc finger-containing transcription factors that are expressed in, e.g., immature hematopoietic cells. An illustration of this class includes members of the GATA family of zinc finger-containing transcription factors, e.g., GATA-1. Inactivation of GATA-1 by homologous recombination causes a decrease in the production of erythroid cells *in vivo* and *ex vivo*, which suggests that this gene may play a role in erythroid development. Other lineage-specific members of the GATA family are also contemplated in this class such as GATA-2, which are expressed in erythroid multipotential progenitors cells and GATA-3, which appears to be expressed predominantly in T lymphoid cells.

Still another source of genes for generating the subject hematopoietic gene constructs include genes encoding certain leucine zipper-containing transcription factors.

An example of this type of developmental control in the hematopoietic system is illustrated with C/EBPβ (also known as NF-IL6, NF-M, LAP, IL-6DBP, AGP/EBP, and CRP2). Expression of C/EBPβ has been shown to change during the differentiation of myeloid cells, consistent with its role as a regulator of differentiation.

As set out above, in a preferred embodiment, the hematopoietic gene construct used to generate the subject genetically-modified stem cells encodes a LIM-homeobox protein, e.g., of the Lhx subclass of homeobox proteins, e.g. *LH-2,* or a homolog thereof. Polypeptides referred to herein as having an activity of an *LH-2* protein preferably have an amino acid sequence corresponding to all or a portion of the amino acid sequence of the human *LH-2* protein shown in SEQ ID No. 2 or 4, or isoforms thereof (including differential splicing variants). It is noted that the *LH-2* sequence shown in SEQ ID Nos. 1 and 2, which clone was used in the experiments described in the appended examples, differs from the published sequence for the human *LH-2* gene (see SEQ ID Nos. 3 and 4) in that the former includes an extra G residue at position of 1653 of SEQ ID No. 1. This extra residue gives rise to a frame shift, resulting in a different c-terminal sequence relative to the published murine homolog. However, the c-terminal sequence of the *LH-2* gene product shown in SEQ ID No. 2 is consistent with the c-terminal sequence published for the chicken and zebrafish *LH-2* clones, and for a rat *LH-2* clone resequenced by Applicant. The sequence difference may be real, i.e. a splicing variant or allelic variation, or merely an error in the published human *LH-2* sequence. In either event, utilizing PCR primers based on the 3' and 5' nucleotide sequence of the human gene will permit isolation of an *LH-2* sequence which will be functional in cells of the instant invention.

LH-2 proteins from other species are also intended to be used as part of this invention, e.g., the rat *LH-2* gene (SwisProt Accession P36198). In preferred embodiments, the biological activity of the ectopically expressed *LH-2* protein includes: an ability to enhance the proliferative potential of a stem cell; an ability to regulate the differentiation potential of a stem cell into hematopoietic lineages, e.g. myeloid, erythroid or lymphoid, in the presence of extracellular factors and cytokines; an ability to create a hematopoietic stem cell that is capable of repopulating the bone marrow upon transplantation.

It will be evident that various homologs of the wild-type *LH-2* protein can also be employed in the cells of the instant invention. In preferred embodiments, the recombinant *LH-2* gene encodes a protein that contains a DNA-binding domain and which comprises an amino acid sequence characterized by Cys-His-rich repeats, e.g., two or more Cys-His rich repeats, e.g., represented in SEQ ID No. 2 or 4. In certain embodiments, the DNA binding domain of an *LH-2* protein can be provided in a fusion protein with a heterologous transcriptional activation domain.

"Homology" and "identity" each refer to sequence similarity between two polypeptide sequences, with identity being a more strict comparison. Homology and identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same amino acid residue, then the polypeptides can be referred to as identical at that position; when the equivalent site is occupied by the same amino acid (e.g., identical) or a similar amino acid (e.g., similar in steric and/or electronic nature), then the molecules can be refered to as homologous at that position. A percentage of homology or identity between sequences is a function of the number of matching or homologous positions shared by the sequences. Sequences which are "unrelated" or "non-homologous" shares less than 40 percent identity, though preferably less than 25 percent identity.

Preferred nucleic acids encode a peptide having an *LH-2* protein activity and being at least 60% homologous, more preferably 70% homologous and most preferably 80% homologous with an amino acid sequence shown in SEQ ID No. 2 or 4. Nucleic acids which encode peptides having an activity of an *LH-2* protein and having at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology with a sequence shown in SEQ ID No. 2 or 4 are also within the scope of the invention. Preferably, the nucleic acid is a cDNA molecule comprising at least a portion of the coding sequence shown in SEQ ID No. 1 or 3.

Modification of the structure of the *LH-2* polypeptide can be for such purposes as enhancing therapeutic or prophylactic efficacy, stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*), or post-translational modifications such as phosphorylation and the like. Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition.

For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (i.e. isosteric and/or isoelectric mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine

histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur -containing = cysteine and methionine. (see, for example, Biochemistry, 2nd ed., Ed. by L. Stryer, WH Freeman and Co.: 1981). Whether a change in the amino acid sequence of a peptide results in a functional *LH-2* homolog (e.g. functional in the sense that the resulting polypeptide mimics the ability of the authentic form to produce the progenitor cells of the present invention) can be readily determined. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

Likewise, the hematopoietic gene construct can be generated with an *LH-2* gene including a nucleotide sequence which hybridizes under high, medium or low stringency conditions to a nucleic acid which encodes an *LH-2* polypeptide comprising Cys-His repeats, e.g., having all or a portion of an amino acid sequence shown in one of SEQ ID No. 1 or 3, e.g., hybridizes to a nucleic acid sequence designated in SEQ ID No. 1 or 3. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology,* John Wiley & Sons, NY. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C.

Nucleic acids which encode biologically active portions of the subject *LH-2* proteins are also within the scope of the invention. As used herein, a fragment of the nucleic acid encoding an active portion of an *LH-2* protein refers to a nucleotide sequence having fewer nucleotides than the nucleotide sequence encoding the full length amino acid sequence of, for example, the *LH-2* proteins represented in SEQ ID No. 1 or 3, and which encodes a peptide which retains transcriptional activity of the full-length protein.

It will be understood that a wide range of vectors, such as described below, can be used for recombinantly expressing hematopoietic genes in stem cells. Such vectors can be constructed using methods well known in the art, including, without limitation, the standard techniques of restriction endonuclease digestion, ligation, plasmid and DNA and RNA purification, DNA sequencing, and the like as described, for example in Sambrook, Fritsch, and Maniatis, eds., Molecular Cloning: A Laboratory Manual., (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. [1989]). Most practitioners are

familiar with the standard resource materials as well as specific conditions and procedures. However, for convenience, the following paragraphs may serve as a guideline.

To generate an appropriate hematopoietic gene construct, nucleic acid corresponding to the intended hematopoietic gene may be obtained from mRNA or genomic DNA present in any of a number of eukaryotic cells in accordance with protocols described herein, as well as those generally known in the art. To illustrate, cDNA encoding an *LH-2* protein can be obtained by isolating total mRNA from a cell expressing that protein, e.g., a hematopoietic or a neural cell. Double stranded cDNAs can then be prepared from the total mRNA, and subsequently inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques. A gene encoding an *LH-2* protein can also be cloned using established polymerase chain reaction techniques in accordance with the nucleotide sequence information provided by the invention.

Any of the methods known to the art for the insertion of DNA fragments into a vector may be used to generate expression constructs of the present invention, including appropriate transcriptional/translational control signals. See, for example, Sambrook et al., *supra*; and Ausubel et al. eds. Current Protocols in Molecular Biology, (John Wiley & Sons, New York [1992]). These methods may include *in vitro* DNA recombinant and synthetic techniques and *in vivo* genetic recombination.

The hematopoietic gene of the present invention will typically be operably linked to transcriptional regulatory sequences, such as promoters and/or enhancers, to regulate expression of the gene in a particular manner. In certain embodiments, the useful transcriptional regulatory sequences are those that are highly regulated with respect to activity, both temporally and spatially. Thus, the promoters of choice can be those that are active only in particular tissues or cell types. Where the promoter is obtained from a mammal, the mammal may be homologous (the same species as the mammal to be transfected) or non-homologous (a different species).

Appropriate promoters/enhancers can be introduced into vectors using standard methods in the art (see e.g., Maniatis). Any promoter that is sufficient to direct the initiation of transcription in a hematopoietic cell may be used in the invention. For example, promoters/enhancers which may be used to control the expression of the recombinant hematopoietic gene include, but are not limited to, the native transcriptional regulatory sequences for the recombinant gene (e.g., the *LH-2* regulatory sequences or the like), the cytomegalovirus (CMV) promoter/enhancer (Keating et al. (1990) *Exp Hematol* 19:99-102;l and Karasuyama et al., 1989, *J Exp. Med.* 169:13), the human β-actin promoter (Gunning et al. (1987) *PNAS* 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al. (1984) *Mol. Cell Biol.* 4:1354-1362), the long terminal repeat sequences of

Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) *RNA Tumor Viruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), promoters fromthe Murine Stem Cell Virus (MSCV) (see Examples below), the SV40 early or late region promoter (Bernoist et al. (1981) *Nature* 290:304-310; Templeton et al. (1984) *Mol. Cell Biol.*, 4:817; and Sprague et al. (1983) *J. Virol.*, 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) *PNAS* 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) *Nature Genetics*, 1 :379-384). A preferred non-tissue specific promoter is the CMV promoter (DeBernardi et al. (1991) PNAS USA 88:9257-61). In this regard, the murine CMV immediate early promoter has been shown to be highly active in human hematopoietic progenitor cells as well as in stromal cells in culture (Keating, A. et al. (1990) Exp. Hematol. 18:99-102).

As an alternative to constitutive expression, the hematopoietic gene may be place under the control of a hematopoietic cell specific promoter. Examples of these promoters include globin promoters, such as the β-globin promoter for expression in erythrocytes (Karlsson et al., (1985) Ann. Rev. Biochem. 54:1071-1108); the Granzyme A promoter for expression in T-cells and NK cells, the CD34 promoter for expression in stem and progenitor cells, the CD8 promoter for expression in cytotoxic T-cells, and the CD11b promoter for expression in myeloid cells.

Inducible promoters may also be used for gene expression under certain physiologic conditions. Those skilled in the art will recognize a variety of inducible eukaryotic promoters which can be used to control expression of the recombinant hematopoietic gene. For example, an IPTG-inducible promoter can be used to conditionally control expression of the recombinant gene. Another transcriptional control system is responsive to hormones (Lee et al. (1981) *Nature* 294:228-232; Hynes et al. (1981) *Proc. Natl. Acad. Sci. USA* 78:2038-2042; Klock et al. (1987) *Nature* 329:734-736; Israel & Kaufman (1989) *Nucl. Acids Res.* 17:2589-2604).

In addition to the hematopoietic gene and the transcriptional regulatory sequence, the vectors useful for preparing the recombinant genes of this invention typically contain one or more other elements useful for optimizing ectopic expression in the host animal. To illustrate, the gene construct may include transcription termination elements, such as to direct polyadenylation of an mRNA transcript, as well as intronic sequences. For example the coding sequence of the recombinant gene can be flanked at its 3' end by SV40 sequences (SV40intron/pA) which add the transcription termination and polyadenylation signals to the transcript. In yet other embodiments, the hematopoietic gene can include intronic sequence(s) interrupting the coding sequence. In many instances, transcription of

a recombinant gene in mammals is increased by the presence of one or more introns in the coding sequence.

In still other embodiments, the gene construct can include additional elements which facilitate its manipulation in cells (e.g., bacterial) prior to insertion in the intended recipient cell. For instance, the vector may include origin of replication elements for amplification in prokaryotic cells.

Moreover, the hematopoietic gene construct can include selectable markers for isolating cells, either from the recipient animal, or generated intermediate the transgenic animal (i.e., bacterial cells used for amplifying the construct). Selectable marker genes can encode proteins necessary for the survival and/or growth of transfected cells under selective culture conditions. Typical selection marker genes encode proteins that, for example: (i) confer resistance to antibiotics or other toxins, e.g., ampicillin, tetracycline or kanomycin for prokaryotic host cells, and neomycin, hygromycin or methotrexate for mammalian cells; or (ii) complement auxotrophic deficiencies of the cell.

B. Systems for Ectopic Expression of the Hematopoietic Gene in Stem Cells

The hematopoietic gene constructs may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively transfecting cells *ex vivo* or *in vivo* with the expression construct. Efficient DNA transfer methods have been developed for hematopoietic cells (see, for example, Keating et al. (1990) *Exp Hematol* 18:99-102; and Dick et al. (1986) *Trends Genet* 2:165) Approaches include insertion of the hematopoietic gene in viral vectors including recombinant retroviruses, adenovirus and adeno-associated virus, or recombinant bacterial or eukaryotic plasmids. Viral vectors can be used to transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramacidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or $CaPO_4$ precipitation carried out. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype, e.g., the degree of commitment the stem cell has undergone, if any. Another factor in the selection of the appropriate transfection formulation is the consideration raised by *ex vivo* transfection versus *in vivo* transfection, with the latter requiring consideration of the route of administration, e.g. locally or systemically.

A preferred approach for both *ex vivo* or *in vivo* introduction of the subject hematopoietic gene construct into a cell is by use of a viral vector containing the

hematopoietic gene. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors are generally understood to be one of the recombinant gene delivery system of choice for the transfer of exogenous genes into stem cells, particularly into humans cells. (see e.g., Hawley R. G., *et al* (1994) *Gene Therapy* 1: 136-38)). These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses as a gene delivery system, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review, see Miller, A.D. (1990*) Blood 76:271*). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence *(gag, pol, env)* has been replaced by the hematopoietic gene, rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *ex vivo* or *in vivo* with such viruses can be found in Ausubel et al., *supra*, Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ΨCrip, ΨCre, Ψ2 and ΨAm.

Retroviruses have been used to introduce a variety of genes into many different cell types, including embryonic stem cells, bone marrow cells, lymphocytes, hepatocytes, by both *ex vivo* and *in vivo* protocols (see for example Eglitis, et al. (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Exemplary retroviral vectors have been described that yield a high titre virus capable of efficiently transducing and expressing genes in undifferentiated embryonic and hematopoietic cells (Hawley *et al* (1994) *Gene Therapy* 1: 136-38). These vectors contain a selectable marker (*neo*, *hph* or *pac*) under the transcriptional control of an internal murine *pgk* promoter and unique restriction sites for insertion of genes downstream of a variant LTR from the retroviral mutant PCMV (PCC4 embryonal carcinoma cell-passaged myeloproliferative sarcoma virus). A variant of the above-described retroviral vectors, the Murine Stem Cell Virus (MSCV), is illustrated in the examples set out below.

In an exemplary embodiment, the entire coding region of the human *LH-2* gene (SEQ. ID. No: 1) is inserted in to the MSCVneo vector (Hawley et al, *supra*) under the control of the viral LTR promoter and also carrying the neomycin phosphotransferase gene as a selectable marker to confer resistance to G418. See Figure 5. Briefly, PCR anchor primers can be used to isolate the human *LH-2* coding sequence and add restriction sites flanking thereto which permit sub-cloning of the *LH-2* sequence into the multiple cloning site of the MSCVneo vector. Helper-free MSCVneo-*LH-2* virus producing packaging cells (see appended examples, and Markowitz et al. (1988) *J Virol* 62:1120-1124) can be made by infection of tunicamycin-treated cells with supernatant from transient transfectants according to the methods of Hawley et al. (1991) *Leukemia Res* 15:659-673. The cells are maintained in, e.g., Dulbecco's modified Eagle medium (DMEM) supplemented with G418. Helper-free MSCVneo-*LH-2* viral stocks can be produced by pooling populations of packaging cells with high titre (e.g., $>10^6$ CFU/ml). The retroviral infection may be performed by either including into the culture medium, supernatants (e.g., 5 to 20% vol/vol) produced by the pooled retroviral packaging cell lines, or by culturing the stem cells directly over the infected retroviral packaging lines themselves, or by both. See, for example, U.S. Patents 5,399,493 and 5,399,346 and PCT publication WO 93/07281.

Returning to the general discussion of retroviral vectors, it is noted that the art demonstrates that it is possible to limit the infective spectrum of retroviruses, and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234, WO94/06920, and WO94/11524). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for stem cell surface antigens to the viral *env* protein (Roux et al. (1989) *PNAS* 86:9079-9083; Julan et al. (1992) *J. Gen Virol* 73:3251-3255; and Goud et al. (1983) *Virology* 163:251-254); or coupling cell surface ligands to the viral *env* proteins (Neda et al. (1991) *J Biol Chem* 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the *env* protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/*env* fusion proteins).

This technique, while useful to limit or otherwise direct the infection to certain tissue types, and can also be used to convert an ecotropic vector in to an amphotropic vector.

To further illustrate, the hematopoietic gene construct can be generated using a retroviral vector which further provides a fusion protein including the viral envelope protein and the vesicular stomatitis virus (VSV-G) glycoprotein (Burns *et al.* (1993) *Proc. Natl. Acad. Sci.* USA 90:8033-37; PCT Patent Application WO 92/14829; and WO 96/09400). Unlike typical amphotropic *env* proteins, the VSV-G protein is thought to mediate viral infection by fusing with a phospholipid component of cell membranes rather than by recognition of a cell surface protein. Since infection is not dependent on a specific receptor, VSV-G pseudotyped vectors have a broad host range. CD34+/Thy-1+ mobilized peripheral blood cells have previously been demonstrated to be transduced with high efficiency by a VSV-G pseudotyped retroviral vector (see Kerr et al. PCT publication WO 96/09400). Genetic modification of the stem cells with a hematopoietic gene construct can be accomplished at any point during their maintenance by transduction with VSV-G pseudotyped virion containing the expression construct.

Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences which control expression of the recombinant hematopoietic gene.

Another viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes the hematopoietic gene product of interest, but is inactivate in terms of its ability to replicate in a normal lytic viral life cycle (see, for example, Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155). Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses are relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity with respect to stem cell populations.

Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al., *supra;* Haj-Ahmand and Graham (1986) J. *Virol.* 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material

(see, e.g., Jones et al. (1979) *Cell* 16:683; Berkner et al., *supra;* and Graham et al. in Methods in Molecular Biology, E.J. Murray, Ed. (Humane, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted hematopoietic gene can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

Yet another viral vector system useful for delivery of the subject hematopoietic genes is the adeno-associated virus (AAV). Adeno-associated viral vectors have been shown to be effective at transducing other genes into pluripotent hematopoietic stem cells *in vitro* (see PCT Application WO 96/08560). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. *Curr. Topics in Micro and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al. (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al. (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to a recombinant hematopoietic gene into stem cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al. (1984) *Proc.. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin et al. (1985) *Mol. Cell. Biol.* 4:2072-2081, Wondisford et al. (1988) *Mol. Endocrinol.* 2:32-39, Tratschin et al. (1984) *J. Virol.* 51:611-619; and Flotte et al. (1993) *J. Biol. Chem.* 268:3781-3790).

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a heterologous hematopoietic gene in transfected stem cells. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the hematopoietic gene construct by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In a representative embodiment, an expression construct including a gene encoding an *LH-2* protein can be entrapped in liposomes bearing positive charges on their surface (e.g., lipofectins) and (optionally) which are tagged with antibodies against cell surface antigens of the targeted stem cell population (Mizuno et al. (1992) *No Shinkei Geka*

20:547-551; PCT publication WO91/06309; Japanese patent application 1047381; and European patent publication EP-A43075).

In yet another illustrative embodiment, the gene delivery system comprises an antibody or cell surface ligand which is cross-linked with a gene binding agent such as polylysine (see, for example, PCT publications W093/04701, W092/22635, W092/20316, WO92/19749, and WO92/06180). It will also be appreciated that effective delivery of the subject nucleic acid constructs via receptor-mediated endocytosis can be improved using agents which enhance escape of the gene from the endosomal structures. For instance, whole adenovirus or fusogenic peptides of the influenza HA gene product can be used as part of the delivery system to induce efficient disruption of DNA-containing endosomes (Mulligan et al. (1993) *Science* 260-926; Wagner et al. (1992) *PNAS* 89:7934; and Christiano et al. (1993) PNAS 90:2122).

For example, the hematopoietic gene construct can be used to transfect hematopoietic stem cells using a soluble polynucleotide carrier comprising a ligand to a stem cell receptor (e.g., steel factor) conjugated to a polycation, e.g. polylysine. To further illustrate, the gene delivery system can be targeted specifically to c-kit-expressing cells, e.g. human hematopoietic progenitor cells. The c-kit protein is a tyrosine kinase receptor for steel factor and is expressed on pluripotential stem cells capable of reconstituting all hematopoietic lineages. Furthermore, c-kit expression is restricted to stem/progenitor cells, and is not expressed on their committed progeny except for expression in mast cells. In an illustrative embodiment, the expression vector for the hematopoietic gene is condensed by electrostatic forces with polylysine (PL) which has been covalently linked to streptavidin and PL which has been covalently linked to adenovirus (in order to achieve endosomal lysis). To the vector-PL conjugate is added a biotinylated steel factor which becomes associated with the vector-PL conjugate through the streptavidin-biotin interaction. Using such constructs, hematopoietic stem cells can be targeted for transfection with the hematopoietic gene construct. See, for example, U.S. Patent 5,166,320, and Schwarzenberger et al., Blood (1996) 87(2): 472-78). One advantage to the PL-vector construct described above is the ability to carry out transient transfection of stem cell populations, while committed hematopoietic cells will be refractory to the transfection process because of a lack of c-kit. Another advantage to this approach derives from the fact that DNA uptake relies on highly efficient receptor-mediated endocytosis, a physiological pathway for macromolecular uptake not associated with cellular toxicity.

The subject hematopoietic gene constructs can be efficiently introduced into stem cells by DNA transfection or by virus-mediated transduction as extensively described above. *In vitro* culturing systems known in the art for stem cells provide an accessible model for genetic manipulations. Possible method of transduction include, but are not

limited to, direct co-culture of stem cells with viral producer cells (see, e.g., Bregni et al. (1992) Blood 80: 1418-22). Alternatively, supernatants from virally infected cells can be isolated and applied to cultures of stem cells under conditions appropriate for infection of the stem cells. See e.g., Xu et al. (1994) Exp. Hemat. 22: 223-30; and Hughes et al. (1992). J. Clin. Invest. 89:1817. The resulting transduced cells may then be grown under conditions similar to those for unmodified stem cells, whereby the modified stem cells may be expanded and caused to differentiate.

In yet another embodiment, the hematopoietic gene can ectopically expressed by use of a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional regulatory sequences of an endogenous form of the gene. For instance, the gene activation construct can replace the endogenous promoter of an *LH-2* gene with a heterologous promoter, e.g., one which causes constitutive expression of the gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of the gene. A variety of different formats for the gene activation constructs are available. See, for example, the Transkaryotic Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

In preferred embodiments, the nucleotide sequence used as the gene activation construct can be comprised of (1) DNA from some portion of the endogenous inductive factor gene (exon sequence, intron sequence, promoter sequences, etc.) which direct recombination and (2) heterologous transcriptional regulatory sequence(s) which is to be operably linked to the coding sequence for the genomic gene upon recombination of the gene activation construct. For use in generating cultures of factor producing cells, the construct may further include a reporter gene to detect the presence of the recombined construct in the genomic DNA of the cell.

The gene activation construct is inserted into a cell, and integrates with the genomic DNA of the cell in such a position so as to provide the heterologous regulatory sequences in operative association with the native gene. Such insertion occurs by homologous recombination, i.e., recombination regions of the activation construct that are homologous to the endogenous inductive factor gene sequence hybridize to the genomic DNA and recombine with the genomic sequences so that the construct is incorporated into the corresponding position of the genomic DNA.

The terms "recombination region" or "targeting sequence" refer to a segment (i.e., a portion) of a gene activation construct having a sequence that is substantially identical to or substantially complementary to a genomic gene sequence, e.g., including 5' flanking sequences of the genomic gene, and can facilitate homologous recombination between the genomic sequence and the targeting transgene construct.

As used herein, the term "replacement region" refers to a portion of a activation construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a recombination region and a genomic sequence.

The heterologous regulatory sequences, e.g., which are provided in the replacement region, can include one or more of a variety elements, including: promoters (such as constitutive or inducible promoters), enhancers, locus control regions, transcription factor binding sites, or combinations thereof. romoters/enhancers which may be used to control the expression of the targeted gene *in vivo* include, but are not limited to, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., 1989, *J. Exp. Med.*, 169:13), the human β-actin promoter (Gunning et al. (1987) *PNAS* 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al. (1984) *Mol. Cell Biol.* 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) *RNA Tumor Viruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) *Nature* 290:304-310; Templeton et al. (1984) *Mol. Cell Biol.*, 4:817; and Sprague et al. (1983) *J. Virol.*, 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) *PNAS* 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) *Nature Genetics*, 1:379-384).

IV. Sources of Cells

Those skilled in the art will appreciate that the subject method can be carried out either in *in vivo* or *ex vivo* (e.g., in cell culture) embodiments. The *in vivo* delivery of a hematopoietic gene construct can be carried out using any of a variety of gene therapy techniques. For *ex vivo* applications, the stem cell to be genetically modified must first be isolated in cell culture. A variety of protocols for isolating embryonic and/or hematopoietic stem cells are well known in the art. Exemplary stem cell cultures for use in the subject method are described below.

A. Isolation of Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) can be isolated from a mammalian source including, but not limited to, bone marrow (both adult and fetal), mobilized peripheral blood (MPB), umbilical cord blood and/or fetal liver. In a preferred embodiment, the HSCs are obtained from the subject into which the stem cells are to be transplanted after *in vitro* culturing and transduction of the hematopoietic gene construct.

The source of cells for the present invention can be, in addition to humans, non-human mammals. A variety of protocols are known in the art for isolating both embryonic stem cells and hematopoietic stem cells from non-human animals. See, for example, the Wheeler U.S. Patent 5,523,226 entitled "Transgenic swine compositions and methods" and the Emery et al. PCT publication WO 95/13363 entitled "Hematopoietic Stem Cells From Swine Cord Blood And Uses Thereof". The preferred non-human animals include vertebrates such as rodents, non-human primates, sheep, dog, cow and pigs. The term "non-human mammal" refers to all members of the class *Mammalia* except humans.

Where the intended use of the resulting hematopoietic cell is for implantation in human patients, the cells derived from transgenic animals can be used as a source for "humanized" hematopoietic cells, e.g., for xenogenic grafting into human subjects. For example, as described by the Sachs et al. PCT publication WO 96/06165 entitled "Genetically Engineered Swine Cells", the art provides for implantation of swine donor cells which have been engineered to increase desirable interactions between the donor cells and molecules and cells of a recipient, e.g., to promote the engraftment or function of the donor stem cells in the recipient environment. To illustrate, the cells can be engineered to express a human adhesion molecule, e.g., an adhesion molecule involved in engraftment and/or maintenance of hematopoietic cells. Examples of human adhesion molecules include VLA-4, c-kit, LFA-1, CD11a, Mac-1, CR3, CD11b, p150, p95, CD11c, CD49a, LPAM-1, CD49d, CD44, CD38, and CD34. The transgenic cells can also be engineered to minimize unwanted interactions between the donor cells and molecules and cells of the recipient which, e.g., promote the rejection of donor graft cells or which inhibit the function of the donor graft cells. For example, the donors cells can be derived from a transgenic animal expressing one or more human MHC polypeptides.

Bone marrow cells can be obtained from a source of bone marrow, including but not limited to, ilium (e.g. from the hip bone via the iliac crest), tibia, femora, spine, or other bone cavities. Other sources of stem cells include, but are not limited to, embryonic yolk sac, fetal liver, and fetal spleen.

For isolation of bone marrow, an appropriate solution can be used to flush the bone, e.g., a salt solution supplemented with fetal calf serum (FCS) or other naturally occurring factors, in conjunction with an acceptable buffer at low concentration, generally from about 5-25 mM. Convenient buffers include HEPES, phosphate buffers and lactate buffers. Otherwise bone marrow can be aspirated from the bone in accordance with conventional techniques.

Methods for mobilizing stem cells into the peripheral blood are known in the art and generally involve treatment with chemotherapeutic drugs, cytokines (e.g. GM-CSF, G-CSF or IL3), or combinations thereof. Typically, apheresis for total white cells begins

when the total white cell count reaches 500-200 cells/l and the platelet count reaches 50,000/l.

Various techniques can be employed to separate the cells by initially removing lineage committed cells. Monoclonal antibodies are particularly useful for identifying markers associated with particular cell lineages and/or stages of differentiation. The antibodies can be attached to a solid support to allow for crude separation. The separation techniques employed should maximize the retention of viability of the fraction to be collected. Various techniques of different efficacy can be employed to obtain "relatively crude" separations. Such separations are where up to 10%, usually not more than about 5%, preferably not more than about 1%, of the total cells present not having the marker can remain with the cell population to be retained. The particular technique employed will depend upon efficiency of separation, associated cytotoxicity, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill.

The use of separation techniques include, but are not limited to, those based on differences in physical (density gradient centrifugation and counter-flow centrifugal elutriation), cell surface (lectin and antibody affinity), and vital staining properties (mitochondria-binding dye rho123 and DNA-binding dye Hoechst 33342). Procedures for separation can include, but are not limited to, magnetic separation, using antibody-coated magnetic beads, affinity chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, including, but not limited to, complement and cytotoxins, and "panning" with antibody attached to a solid matrix, e.g., plate, elutriation or any other convenient technique. Techniques providing accurate separation include, but are not limited to, FACS, which can have varying degrees of sophistication, e.g., a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, etc.

A large proportion of the differentiated cells can be removed by initially using a relatively crude separation, where major cell population lineages of the hematopoietic system, such as lymphocytic and myelomonocytic, are removed, as well as minor populations, such as megakaryocytic, mast cells, eosinophils and basophils. Usually, at least about 70 to 90 percent of the hematopoietic cells will be removed. If desired, a prior separation can be employed to remove erythrocytes, by employing Ficoll-Hypaque separation.

The gross separation can be achieved using methods known in the art including, but not limited to, magnetic beads, cytotoxic agents, affinity chromatography or panning. Antibodies which find use include antibodies to lineage specific markers which allow for removal of most, if not all, mature cells, while being absent on stem cells.

Concomitantly or subsequent to a gross separation, which provides for positive selection, a negative selection can be carried out, where antibodies to lineage-specific markers present on dedicated cells are employed. For the most part, these markers include, but are not limited to, CD2-, CD3-, CD7-, CD8-, CD10-, CD14-, CD15-, CD16-, CD19-, CD20-, CD33- and glycophorin A; preferably including, but not limited to, at least CD2-, CD14-, CD15-, CD16-, CD19- and glycophorin A; and normally including at least CD14- and CD15-. As used herein, Lin refers to a cell population lacking at least one lineage-specific marker. The hematopoietic cell composition substantially depleted of dedicated cells can then be further separated using a marker for Thy-1, whereby a substantially homogeneous stem cell population is achieved. Exemplary of this stem cell population is a population which is CD34+Thy-1+Lin-, which provides an enriched stem cell composition. Other markers that have been reported to subdivide CD34+ cells, further enriching for stem cells include, but are not limited to, CD38-, rhodamine lo, c-kit receptor, HLA DR lo/-, CD71, and CD45 RA-. In fetal tissues and umbilical cord, stem cells are highly enriched in the CD34 hiLin- populations as described by Giusto et al. (1993) Blood 84: 421-32.

The purified stem cells have low side scatter and low to medium forward scatter profiles by FACS analysis. Cytospin preparations show the enriched stem cells to have a size between mature lymphoid cells and mature granulocytes. Cells can be selected based on light-scatter properties as well as their expression of various cell surface antigens.

While it is believed that the particular order of separation is not critical to this invention, the order indicated is preferred. Preferably, cells are initially separated by a coarse separation, followed by a fine separation, with positive selection of a marker associated with stem cells and negative selection for markers associated with lineage committed cells.

Compositions highly enriched in stem cells can be achieved in this manner. The desired stem cells are exemplified by a population with the CD34+Thy-1+Lin- phenotype and being able to provide for cell regeneration and development of members of all of the various hematopoietic lineages.

It should be noted that negative selection lineage selection for lineage specific markers provide a greater enrichment in stem cells obtained from bone marrow than from MPB. The majority of CD34 cells that are mobilized into the peripheral blood do not express lineage-specific markers and, therefore, Lin selection does not significantly enrich over CD34 selection in the peripheral blood as it does in bone marrow. Selection for Thy-1+ does enrich for stem cells in both mobilized peripheral blood and bone marrow.

Fetal or neonatal blood are also sources for the hematopoietic stem and progenitor cells of the present invention.

Fetal blood can be obtained by any method known in the art. For example, fetal blood can be taken from the fetal circulation at the placental root with the use of a needle guided by ultrasound (Daffos et al., (1985) *Am. J. Obstet Gynecol* 153:655-660; Daffos et al., (1983) *Am. J. Obstet. Gynecol.* 146:985), by placentocentesis (Valenti, C., (1973) *Am. J. Obstet. Gynecol.* 115:851; Cao et al., (1982) *J. Med. Genet.* 19:81), by fetoscopy (Rodeck, C.H., (1984) in Prenatal Diagnosis, Rodeck, C.H. and Nicolaides, K.H., eds., Royal College of Obstetricians and Gynaecologists, London), etc.

In a preferred embodiment of the invention, neonatal hematopoietic stem and progenitor cells can be obtained from umbilical cord blood and/or placental blood. The use of cord or placental blood as a source of hematopoietic cells provides numerous advantages. Cord blood can be obtained easily and without trauma to the donor. In contrast, at present, the collection of bone marrow cells is a traumatic experience which is costly in terms of time and money spent for hospitalization. Cord blood cells can be used for autologous transplantation, when and if needed, and the usual hematological and immunological problems associated with the use of allogeneic cells, matched only partially at the major histocompatibility complex or matched fully at the major, but only partially at the minor complexes, are alleviated.

Collections should be made under sterile conditions. Immediately upon collection, the neonatal or fetal blood should be mixed with an anticoagulent. Such an anticoagulent can be any known in the art, including but not limited to CPD (citrate-phosphate-dextrose), ACD (acid citrate-dextrose), Alsever's solution, De Gowin's Solution , Edglugate-Mg, Rous-Turner Solution, other glucose mixtures, heparin, ethyl biscoumacetate, etc. (See Hurn, B.A.L., 1968, Storage of Blood, Academic Press, New York, pp. 26-160).

B. Isolation of Embryonic Stem Cells

The present system is based on the ability of ES cells to differentiate and generate hematopoietic cells in culture and *in vivo*, e.g., to recapitulate hematopoiesis. Previous studies have demonstrated that ES cells will differentiate in culture and generate multiple hematopoietic lineages. However, in most of these studies, the extent of hematopoietic development has been limited and variable, and the exact kinetics of hematopoietic differentiation has been unpredictable or poorly defined. Utilizing the subject method, hematopoietic stem cells can be generated by ectopic expression of a hematopoietic gene such as *LH-2*. The advantages of such a system are several-fold. First, one has access to the cells at all stages of differentiation, making it possible to manipulate the system as it develops. Second, an *in vitro* system based on ES cells will enable one to study the function of a broad spectrum of genes through inactivation by homologous recombination

without encountering the problems inherent to an *in vivo* system; namely, embryonic lethalities.

Embryonic stem cells are generated and maintained using methods well known to the skilled artisan such as those described by Weiss et al. (1996) *J Clinical Inves* 97:591-595; and Doetschman et al. (1985) *J. Embryol. Exp. Morphol.* 87:27-45). Any line of ES cells can be used, however, the line chosen is typically selected for the ability of the cells to differentiate into embryoid bodies (EB) followed by their commitment into hematopoietic lineages, e.g. erythroid, lymphoid, myeloid. Thus, any ES cell line from human or non-human origin that is believed to have this capability is suitable for use herein. As an example of one mouse strain that is typically used for production of ES cells, is the 129J strain, e.g. cell line CCE utilized in the Examples below. Still another preferred murine cell line is the cell line J1. Other ES cell lines include D3 (American Type Culture Collection, catalog no. CKL 1934) and the WW6 cell line (see Ioffe et al. (1995) *PNAS* 92:7357-7361).

ES cells are cultured using methods well known to the skilled artisan, such as those set forth by Robertson in: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, E.J. Robertson, ed. IRL Press, Washington, D.C. [1987]); by Bradley et al. (1986) *Current Topics in Devel. Biol.* 20:357-371); and by Hogan et al. (Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY [1986]). As an illustration, ES cells can be grown and passaged *in vitro* with or without feeder layers, e.g., embryonic fibroblasts, in the presence of growth factors selected from steel factor (membrane -associated or soluble forms), leukemia inhibitory factor (LIF) and fibroblast growth factor (FGF). See e.g., US Pat No. 5,453,357. Growth and differentiation enhancing concentrations of these factors can range from 0.5-500 ng/ml, preferably, 10-20 ng/ml. Differentiation of ES cells into embryoid bodies (EBs) and multiple cell-types, e.g., hematopoietic, endothelial, muscle and neuronal lineages, can be achieved by a number of standard methods known in the art (reviewed in G. Keller, Current Opinion: 862-69; see also, G. Keller et al. *MCB* 13(1) 473-86).

The technique most frequently used to differentiate ES cells is simply to remove the cells from contact with the feeder cells, or from the presence of LIF, and culture them in liquid or methyl cellulose containing media in bacterial grade petri dishes. Under these conditions, ES cells are unable to adhere to the surface of the dish, and the formation of EBs is enhanced. A modification of this method to maximize ES cell differentiation into hematopoietic lineages involves the culturing of these cells on stromal cells, which provides a supportive environment for hematopoietic cells as they develop within the EBs. Stromal cell culturing methods are extensively described below in *Expansion and Differentiation of Genetically Modified Cells*. Once EBs are formed, they can be

dissociated into a single cell suspension. The generated EBs can be assayed at various stages of development for the presence of specific cell populations. For example, hematopoietic lineages can be examined by plating EB-derived cells in methyl cellulose in the presence of growth factors for determining hematopoietic precursor populations. A specific illustration of precursor cell assay is provided in the Examples below. In brief, single cell suspension of EBs can be assayed for precursor content in colony forming cell-culture (CFC-c) assay as described in Keller et al., *supra*. This procedure will be hereinafter referred to as the precursor assays. Alternatively, EB-derived cells can be analyzed for the presence of specific cell-surface antigens (e.g., immunoglobulin can be used to stain B lymphocytes) by immunocytochemical methods or by FACS analysis. The most stringent test for the differentiation potential of these cells involves the ability of dissociated EB cells to repopulate the hematopoietic system of a recipient animal. Detection of cell surface antigens and transplantation protocols are extensively described below in the section entitled "*Expansion and Differentiation of Genetically Modified Cells*".

V. Expansion and Differentiation of Genetically Modified Stem Cells

In general, the stem cells used for transfection can be cultured, both before and after modification with the hematopoietic gene construct, by standard protocols known in the art. For instance, the cells can be cultured in defined or serum-derived media alone, or as co-cultures with a feeder cell layer, e.g., a stromal cell co-culture system. The isolated stem cells can be cultured in minimal essential medium supplemented with serum and antibiotics. Culture media that can be used include, for example, Hanks. McCoys, RPMI 1640 minimal essential media (MEM), and others, and include 1% to 20% serum. Extracellular factors and cytokines that can de added are described below in the section *Expansion and Differentiation of Stem Cells*. While the subject stem cells can be grown in complex media, it will generally be preferred that the cells be maintained in a simple medium, such as Dulbecco's Minimal Essential Media (DMEM), in order to effect more precise control over the activation of certain progenitor populations in the culture.

The cells may be maintained in any suitable culture vessel, such as a 12 or 24 well microplate, and may be maintained under typical culture conditions for cells isolated from the same animal, e.g., such as 37°C in 5% CO_2.

In yet other embodiments, the modified stem cells can be cultured on feeder layers, e.g., layers of feeder cells which secrete inductive factors or polymeric layers containing inductive factors. In preferred embodiments, the cells are expanded in a stromal cell co-culture system. See, for example, Deryugina et al. (1993) *Crit Rev Immunology* 13:115-150. Stromal cells are believed to provide not only a physical matrix on which stem cells

reside, but also to produce membrane-contact signals and/or hematopoictic growth factors which can augment stem cell proliferation and differentiation. A variety of different stromal culture systems are available. In general, the modified stem cells are cultured on the stromal layer, and non-adherent cells isolated for further passage, implantation or other use.

In an exemplary embodiment, the subject stem cells can be supported in the long term cultures developed by Dexter and co-workers (Dexter et al. (1977) *J Cell Physiol* 91:335; and Dexter et al. (1979) *Acta Haematol* 62:299). It is relatively easy to establish Dexter long term cultures from human marrow. However, in contrast to murine marrow cells, hematopoiesis in human Dexter cultures has previously been limited to about 2 to 3 months. It is anticipated that the modified human stem cells of the present invention will have significantly lengthened lifespan in the Dexter culture.

Yet another stromal layer which can be used to support the modified stem cells of the present invention is derived from bone marrow endothelial cells. See, for example, Asch et al. PCT publication WO96/00779. Bone marrow endothelial cells are isolated by a process which involves a bone marrow aspirate containing spicules including vessel fragments. Bone marrow spicules are recovered from the marrow, and digested with proteolytic enzymes. Mircovessel fragments from the digested spicules are recovered, and these fragments grown as explants to form bone marrow endothelial cells.

Moreover, a variety of mammalian stromal cell lines are available and can be used to generate a confluent cell layer upon which the instant stem cell preparations can be cultured. Exemplary human stromal cell lines include KM-102, SV-MSC, ST-1, SCL and H-7 cell lines (see, for example, Deryugina et al., *supra*).

In another embodiment, a matrigel layer or the like can be used to induce expansion of the stem cell population. Matrigel (Collaborative Research, Inc., Bedford, Mass.) is a complex mixture of matrix and associated materials derived as an extract of murine basement membrane proteins, consisting predominantly of laminin, collagen IV, heparin sulfate proteoglycan, and nidogen and entactin was prepared from the EHS tumor as described Kleinman et al, "Basement Membrane Complexes with Biological Activity", Biochemistry, Vol. 25 (1986), pages 312-318. Other such matrixes can be provided, such as Humatrix.

Likewise, natural and recombinantly engineered cells can be provided as feeder layers to the instant cultures.

Still other culture systems include the three-dimensional stromal cell and tissue culture system of the Slivka et al. U.S. patent 5,478,739 and the like.

In other embodiments, the stem cells of the instant invention can be cultured in "stroma-free" cell culture media which is supplemented with such cytokines as, for example, Il-3, LIF, G-CSF and SCF. As described by McGlave et al., U.S. Patent 5,523,286, stem cells can be co-cultured with a stroma-derived anionic fraction in combination with cytokines. According to that method, the anionic fraction is prepared by a process comprising subjecting stroma cell conditioned aqueous culture medium to ion-exchange chromatography so as to isolate the anionic glycoprotein fraction having a molecular weight of greater than 200 kD. It is believed that the bioactive anionic fraction comprises a mixture of glycoproteins, including proteoglycans.

B. Differentiation Factors

The differentiation pathway and growth factor dependency of hematopoietic stem cells are well documented in the art. Pluripotent HSCs gain and lose antigenic features and responsiveness to specific cytokines based on their lineage and stage of differentiation (Figure 1). The genetically-modified stem cells of the present invention can be characterized in terms of their growth factor responsiveness and cell surface antigens expressed. The teachings illustrated in Figure 1 are incorporated in the method of characterizing and differentiating pluripotent HSCs into myeloid or lymphoid progenitors described in the present invention.

For example, any combination of known hematopoietic factors, e.g., extracellular factors and cytokines, can be added to instant stem cell cultures *ex vivo* and changes in cell responsiveness can be assessed, e.g., changes in the signal transduction cascade and/or cell growth or differentiation. The factors that can be used include, but is not limited to, erythropoietin (EPO), thrombopoietin, granulocyte/macrophage colony stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), interleukins 1 -12 (IL-1 to IL-12) and steel factor (SF). Of particular interest are LIF, steel factor, erythropoietin, IL-3, IL-6, GM-CSF and G-CSF. These factors can be added in isolation or in any combination. The factors which are employed may be naturally occurring or synthetic, e.g., prepared recombinantly, and may be human or non-human, e.g., murine. The amount of the factors will generally be in the range of about 1ng/ml to 100 ng/ml per day. Generally, for steel factor the concentration will be in the range of about1 to 100 ng/ml; for LIF, the concentration will be in the range of about 1 ng/ml to 100 ng/ml, more usually 5 ng/ml to 30 ng/ml; for IL-3, the concentration will be in the range of about 5 ng/ml to 500 ng/ml, more usually 5 ng/ml to 100 ng/ml; for IL-6, the concentration will be in the range of about 5 ng/ml to 50 ng/ml, more usually 5 ng/ml to 20 ng/ml; for Erythropoietin, the concentration will be in the

range of about 1 to 10 units/mL; and for GM-CSF, the concentration will generally be 5 ng/ml to 50 ng/ml, more usually 5 ng/ml to 20 ng/ml.

In one embodiment, the stem cells are optionally expanded prior to or after transduction. During expansion, the growth factors may be present only during the initial course of the stem cell growth and expansion, usually at least 24 hours, more usually at least 48 hours to 4 days or may be maintained during the course of the expansion.

For clinical settings, it is preferable to transduce the stem cells without prior or subsequent expansion. In one embodiment therefore, the stem cells are cultured with or without cytokines in an appropriate medium, transduced with the appropriate vector, cultured for approximately 72 hours and reintroduced into the host.

Furthermore, monoclonal or polyclonal antibodies can be used to identify cell surface antigens associated with particular cell lineages and/or stages of differentiation. As an illustration, cell surface antigens for the following lineages can be followed: a pluripotent HSC, e.g. cells having the antigenic phenotype of CD34+, CD38- can become sequentially restricted to myeloid lineages, e.g., burst-forming units-erythroid (BFU-E) to a colony forming unit-erythroid (CFU-E); a burst-forming units-megakaryocyte (BFU-Meg) to a colony forming unit-megakaryocyte (CFU-Meg); a colony forming unit-granulocyte-monocyte (CFU-GM) to a colony forming unit-eosinophil (CFU-Eo), or a colony forming unit-granulocyte-erythrocyte-megakaryocyte-monocyte (CFU-GEMM). Alternatively, a pluripotent HSC can become sequentially restricted to lymphoid lineages, e.g., a lineage that gives rise to T-or B-cell progenitor cells.

C. Characterization and Isolation of the Genetically-Modified Hematopoietic Stem Cells

Various techniques may be employed to characterize and isolate either the genetically modified stem cells or the differentiated progeny thereof. Preferred isolation procedures are the ones that result in as little cell death as possible. For example, isolation of a particular differentiated cell population can be accomplished by relying on expression patterns of specific cellular markers, e.g., using affinity separation techniques or fluorescence activated cell sorting (FACS).

To further illustrate, monoclonal antibodies are particularly useful for identifying markers (surface membrane proteins, e.g., receptors) associated with particular cell lineages and/or stages of differentiation. Procedures for separation of a cell of particular hematopoietic phenotype may include, as described above for the isolation of the stem cell culture, such techniques as magnetic separation, using antibody coated magnetic beads, affinity chromatography, and "panning" with antibody attached to a solid matrix, e.g., plate, or other convenient technique. Techniques providing accurate separation include

fluorescence activated cell sorting, which can have varying degrees of sophistication, e.g., a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, etc.

Conveniently, the antibodies may be conjugated with markers, such as magnetic beads, which allow for direct separation, biotin, which can be removed with avidin or streptavidin bound to a support, fluorochromes, which can be used with a fluorescence activated cell sorter, or the like, to allow for ease of separation of the particular cell type. Any technique may be employed which is not unduly detrimental to the viability of the cells.

Upon isolation, the cells can be further characterized in the following manner: responsiveness to growth factors, specific gene expression, antigenic markers on the surface of such cells, and/or basic morphology. For example, extent of growth factor responsivity, e.g., the concentration range of growth factor to which they will respond to, the maximal and minimal responses, and to what other growth factors and conditions to which they might respond, can be used to characterize the hematopoietic cell. Furthermore, the isolated progenitor cells can be characterized by the expression of genes known to mark the commitment to a particular lineage.

The art provides a variety of assays for determining if a stem cell is a hematopoietic stem cell. The most stringent experimental criterion for hematopoietic stem cells is the ability of these cells to reconstitute the entire blood system of a lethally irradiated animals. The most widely accepted assay for human HSCs is the Long Term Culture-Initiating Cell (LTC-IC) assay, which involves culturing bone marrow cells for 5 weeks and then analyzing the developmental potential of surviving cells by plating these cells in semi-solid medium (Eaves et al., (1991) J. Tiss. Cult. Meth. 13:55-62). After 14 days in semi-solid medium, colonies of all cell lineages are detectable if pluripotent, self-renewing cells were present when the long term culture was initiated. In assays for LTCIC, the highly enriched stem cell population will typically have an LTCIC frequency in the range of 1/20 to 1/100; preferably it will have a frequency of at least 1/50. Animal models for long term engrafting potential include the SCID-hu bone model and the in utero sheep model. For review, see Srour et al. (1992), J. Hematother. 1: 143-53.

D. Pharmaceutical Preparation of Cells

Yet another aspect of the present invention concerns cellular compositions which include, as a cellular component, substantially pure preparations of the subject genetically modified stem cells, or the progeny thereof. Cellular compositions of the present invention include not only substantially pure populations of the stem cells, but can also

include cell culture components, e.g., culture media including amino acids, metals, coenzyme factors, as well as small populations of non-stem cells, e.g., some of which may arise by subsequent differentiation of isolated stem cells of the invention. Furthermore, other non-cellular components include those which render the cellular component suitable for support under particular circumstances, e.g., implantation, e.g., continuous culture.

As common methods of administering the stem cells of the present invention to subjects, particularly human subjects, which are described in detail herein, include injection or implantation of the cells into target sites in the subjects, the cells of the invention can be inserted into a delivery device which facilitates introduction by, injection or implantation, of the cells into the subjects. Such delivery devices include tubes, e.g., catheters, for injecting cells and fluids into the body of a recipient subject. In a preferred embodiment, the tubes additionally have a needle, e.g., a syringe, through which the cells of the invention can be introduced into the subject at a desired location. The stem cells of the invention can be inserted into such a delivery device, e.g., a syringe, in different forms. For example, the cells can be suspended in a solution or embedded in a support matrix when contained in such a delivery device. As used herein, the term "solution" includes a pharmaceutically acceptable carrier or diluent in which the cells of the invention remain viable. Pharmaceutically acceptable carriers and diluents include saline, aqueous buffer solutions, solvents and/or dispersion media. The use of such carriers and diluents is well known in the art. The solution is preferably sterile and fluid to the extent that easy syringability exists. Preferably, the solution is stable under the conditions of manufacture and storage and preserved against the contaminating action of microorganisms such as bacteria and fungi through the use of, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. Solutions of the invention can be prepared by incorporating stem cells as described herein in a pharmaceutically acceptable carrier or diluent and, as required, other ingredients enumerated above, followed by filtered sterilization.

Support matrices in which the stem cells can be incorporated or embedded include matrices which are recipient-compatible and which degrade into products which are not harmful to the recipient. Natural and/or synthetic biodegradable matrices are examples of such matrices. Natural biodegradable matrices include plasma clots, e.g., derived from a mammal, and collagen matrices. Synthetic biodegradable matrices include synthetic polymers such as polyanhydrides, polyorthoesters, and polylactic acid. Other examples of synthetic polymers and methods of incorporating or embedding cells into these matrices are known in the art. See e.g., U.S. Patent No. 4,298,002 and U.S. Patent No. 5,308,701. These matrices provide support and protection for the fragile stem cells *in vivo* and are, therefore, the preferred form in which the stem cells are introduced into the recipient subjects.

VI. Exemplary Uses of the Stem Cells

The genetically modified stem cells of the invention, and the progeny thereof, can be used in a variety of applications. These include but are not limited to transplantation or implantation of the engineered cells *in vivo*; screening cytotoxic compounds, allergens, growth/regulatory factors, pharmaceutical compounds, etc., *in vitro*; elucidating the mechanism of certain diseases; studying the mechanism by which drugs and/or growth factors operate; and the production of biologically active products, to name but a few.

For instance, the present invention provides substantially pure stem cells which can be used therapeutically for treatment of illnesses requiring bone marrow transplantation, such as all cancer treatments involving radio- or chemotherapy, ii) a possible cure for auto immune diseases by replacing pathogenic lymphocytes iii) a possible cure for different kinds of inherited or acquired anemias, iv) AIDS if the possibility exists that the HIV infected compartment can be replaced with healthy cells by expanding a small number of the patients own non-infected stem cells.

A. Drug Screening

The engineered stem cells of the present invention may be used *in vitro* to screen a wide variety of compounds, such as cytotoxic compounds, growth/regulatory factors, pharmaceutical agents, etc. To this end, the cultures are maintained *in vitro* and exposed to the compound to be tested. The activity of a cytotoxic compound can be measured by its ability to damage or kill cells in culture. This may readily be assessed by vital staining techniques. The effect of growth/regulatory factors may be assessed by analyzing the cellular content of the culture, e.g., by total cell counts, and differential cell counts. This may be accomplished using standard cytological and/or histological techniques including the use of immunocytochemical techniques employing antibodies that define type-specific cellular antigens. The effect of various drugs on the cells may be assessed. For example, drugs that increase red blood cell formation can be tested.

B. Transplantation

Disorders that can be treated by infusion of stem cells include but are not limited to five broad categories. First are diseases resulting from a failure or dysfunction of normal blood cell production and maturation (i.e., aplastic anemia and hypoproliferative stem cell disorders). The second group are neoplastic, malignant diseases in the hematopoietic organs (e.g., leukemia and lymphomas). The third group of disorders comprises those of

patients with a broad spectrum of malignant solid tumors of non-hematopoietic origin. Stem cell infusion in these patients serves as a bone marrow rescue procedure, which is provided to a patient following otherwise lethal chemotherapy or irradiation of the malignant tumor. The fourth group of diseases consists of autoimmune conditions, where the stem cells serve as a source of replacement of an abnormal immune system. The fifth group of diseases comprises a number of genetic disorders which can be corrected by infusion of hematopoietic stem cells, preferably syngeneic, which prior to transplantation have undergone gene therapy. Particular diseases and disorders which can be treated by hematopoietic reconstitution with neonatal stem and progenitor cells include but are not limited to those described below. Diseases resulting from a failure or dysfunction of normal blood cell production and maturation, including: hyperproliferative stem cell disorders, aplastic anemia, pancytopenia, agranulocytosis, thrombocytopenia, red cell aplasia, Blackfan-Diamond syndrome, due to drugs, radiation, or infection, idiopathic. Hematopoietic malignancies including: acute lymphoblastic (lymphocytic) leukemia, chronic lymphocytic leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, acute malignant myelosclerosis, multiple myeloma, polycythemia vera, agnogenic myelometaplasia, Waldenstrom's macroglobulinemia, Hodgkin's lymphoma, non-Hodgkins's lymphoma. Immunosuppression in patients with malignant, solid tumors, such as: malignant melanoma, carcinoma of the stomach, ovarian carcinoma, breast carcinoma, small cell lung carcinoma, retinoblastoma, testicular carcinoma, glioblastoma, rhabdomyosarcoma, neuroblastoma, Ewing's sarcoma, lymphoma. Autoimmune diseases such as: rheumatoid arthritis, diabetes type I, chronic hepatitis, multiple sclerosis, systemic lupus erythematosus. Genetic (congenital) disorders, including: anemias, familial aplastic, Fanconi's syndrome, Bloom's syndrome, pure red cell aplasia (PRCA), dyskeratosis congenita, Blackfan-Diamond syndrome, congenital dyserythropoietic syndromes I-IV, Swachmann-Diamond syndrome, dihydrofolate reductase deficiencies, formamino transferase deficiency, Lesch-Nyhan syndrome, congenital spherocytosis, congenital elliptocytosis, congenital stomatocytosis, congenital Rh null disease, paroxysmal nocturnal hemoglobinuria, G6PD (glucose-6-phosphate dehydrogenase), variants 1,2,3, pyruvate kinase deficiency, congenital erythropoietin sensitivity, deficiency, sickle cell disease and trait, thalassemia alpha, beta, gamma, met-hemoglobinemia, congenital disorders of immunity, severe combined immunodeficiency disease (SCID), bare lymphocyte syndrome, ionophore-responsive combined immunodeficiency, combined immunodeficiency with a capping abnormality, nucleoside phosphorylase deficiency, granulocyte actin deficiency, infantile agranulocytosis, Gaucher's disease, adenosine deaminase deficiency, Kostmann's syndrome, reticular dysgenesis, congenital leukocyte dysfunction syndromes. Others disorders which may treated with the hematopoietic cells of the present invention include: osteopetrosis, myelosclerosis, acquired hemolytic

anemias, acquired immunodeficiencies, infectious disorders causing diseases, mucopolysaccharidoses, mucolipidoses, miscellaneous disorders involving immune mechanisms, Wiskott-Aldrich Syndrome, alpha 1-antitrypsin deficiency.

In addition to allowing a more effective purge by treating smaller volumes of diseased marrow followed by expansion *in vitro*, the three-dimensional culture system can be utilized on larger volumes of purged marrow. A side effect of most purging agents is destruction and disruption of normal hematopoietic skin cells, which results in a prolonged time to engraftment and often patient mortality due to secondary infection. One effective purging agent utilized with acute nonlymphocytic leukemia is 4-hydroperoxyoyolo phosphamide (4HC) which causes a two log kill of malignant cells. In traditional treatment, 500 ml-1000 ml of diseased marrow is treated by incubation of the marrow *ex vivo* with 60-100 ng of 4HC/ml. Marrow is then cryopreseved and reinfused into the patient after 2-3 weeks of clinical chemotherapy. According to the present invention, a comparable volume of bone marrow may be harvested, purged with 4HC, and then expanded *in vitro* in three-dimensional culture, thereby allowing a more rapid engraftment time and a decrease in patient mortality.

The stem cell cultures of the present invention may be used for treating diseases or conditions which destroy healthy bone marrow cells or depress their functional ability. The process is effective especially in the treatment of hematological malignancies and other neoplasias which metastasize to the bone marrow. This aspect of the invention is also effective in treating patients whose bone marrow has been adversely affected by environmental factors, (e.g., radiation, toxins etc.), chemotherapy and/or radiation therapy necessitated by a disease which does not directly affect the bone marrow. In these cases, for example, bone marrow cells from a healthy patient can be removed, preserved, and then replicated and reinfused should the patient develop an illness which either destroys the bone marrow directly or whose treatment adversely affects the marrow. The stem cell culture system of the present invention has several advantages to a patient in need of a bone marrow transplant. If the patient is receiving his or her own cells, this is called an autologous transplant; such a transplant has little likelihood of rejection. Autologous transplants eliminate a major cause of bone marrow transplant rejection, that is, the graft vs. host reaction. If the marrow contains malignant or diseased cells, small samples of it can be more effectively purged and the stem cells expanded using the culture system of the invention. As understood in the art, selective methods for purging malignant or diseased cells work best in small volumes of bone marrow cells. The stem culture system described herein makes this feasible. Accordingly, a small sample obtained from the patient can be more efficiently purged using a selective method that kills malignant cells yet spares healthy cells. The remaining healthy cells can then be expanded considerably using the subject technique.

According to the present invention, a relatively small volume of bone marrow from a diseased patient may be harvested and the patient's bone marrow destroyed by chemotherapy or radiation. The bone marrow sample may then be purged of diseased cells using an appropriate chemotherapeutic agent, transfected with a hematopoietic gene construct according to the method of the present invention, the genetically modified cells expanded *in vitro*, and then readministered to the patient.

In another embodiment, the genetically modified cells of the present invention can be used in the treatment of AIDS related conditions. According to an abstract submitted by the authors to the 37th Annual Meeting of the American Society of Hematology, held December 1-5, 1995, in Seattle, Washington, "Cytopenias are a common complication of human immunodeficiency virus (HIV) infection and can affect different hematopoietic lineages including erythropoiesis, lymphopoiesis, thrombopoiesis, and granulopoiesis". Those authors quantitated by flow cytometry the primitive cells of bone marrow aspirates of 23 patients with AIDS. Although the mean percentage of CD34(+) was comparable to normal adult bone marrow, phenotype analysis of CD34(+) populations showed that the CD34(+)/CD38(-), CD34(+)/CD381CD4(+) and CD34(+)1HY-I(+) subsets were severely reduced in all patients in comparison to normal donors, suggesting a significant loss of immature hematopoietic progenitors. *In vitro* functional analysis of the sorted CD34(+) from HIV-1 patients in long-term culture-initiating cell (LTC-IC) assays showed a high decrease in clonogenic activity when compared to controls. In order to more accurately quantitate the LTC-IC frequency, CD34(+) cells from three patients were then sorted in a limiting dilution assays. Colony formation was assessed five weeks later in individual wells and the percentage of negative wells was used to calculate the LTC-IC frequency. Using this approach, those researchers found that the frequency of LTC-IC in CD34(+) derived-HIV-1 patients is approximately 7 to 10-fold lower than that measured in normal marrow aspirate samples. Since the majority of normal primitive progenitors expresses CM, infection of immature progenitors was investigated both by studying long-term culture derived individual colonies or in purified suspension of CD34(+) cells using the PCR technique. HIV-1 DNA was absent from CD34(+) cells in six patients examined indicating that others mechanisms than direct viral infection of immature hematopoietic progenitors must account for the defective hematopoiesis in HIV-1 infected patients. In conclusion, the data presented a quantitative deficiency of immature progenitor cell subset in AIDS patients.

The present invention provides a source of hematopoietic stem cells, or the progeny thereof, which can be used to augment performance of the hematopoietic system of an AIDS patient. In preferred embodiments, hematopoietic stem cells are isolated from the patient (or a matched donor) and, as necessary, purged of virally infected cells. The stem cells are than transfected with a hematopoietic gene construct according to the

present method. The resulting cells are expanded in culture, and either implanted in the patient, or further differentiated into one or more hematopoietic lineages which are then implanted in the patient. It will be apparent from the present description that transfection of the stem cells can alternatively be carried *in vivo* as part of a gene therapy approach.

C. Modification of Stem Cells In vivo

In yet another embodiment, the hematopoietic gene construct can be used to transfect cells *in vivo*, e.g., as part of a gene therapy protocol. In clinical settings, the hematopoietic gene delivery systems can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the in the target stem cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) PNAS 91: 3054-3057).

The hematopoietic gene delivery system can be provided as a pharmaceutical preparation, .e.g., in an acceptable diluent or as part of a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral packages, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system. In the case of the latter, methods of introducing the viral packaging cells may be provided by, for example, rechargeable or biodegradable devices. Various slow release polymeric devices have been developed and tested *in vivo* in recent years for the controlled delivery of drugs, including proteinacious biopharmaceuticals, and can be adapted for release of viral particles through the manipulation of the polymer composition and form. A variety of biocompatible polymers (including hydrogels), including both biodegradable and non-degradable polymers, can be used to form an implant for the sustained release of an the viral particles by cells implanted at a particular target site. Such embodiments of the present invention can be used for the delivery of an exogenously purified virus, which has been incorporated in the polymeric device, or for the delivery of viral particles produced by a cell encapsulated in the polymeric device.

By choice of monomer composition or polymerization technique, the amount of water, porosity and consequent permeability characteristics can be controlled. The

selection of the shape, size, polymer, and method for implantation can be determined on an individual basis according to the disorder to be treated and the individual patient response. The generation of such implants is generally known in the art. See, for example, Concise Encyclopedia of Medical & Dental Materials, ed. by David Williams (MIT Press: Cambridge, MA, 1990); and the Sabel et al. U.S. Patent No. 4,883,666. In another embodiment of an implant, a source of cells producing a the recombinant virus is encapsulated in implantable hollow fibers. Such fibers can be pre-spun and subsequently loaded with the viral source (Aebischer et al. U.S. Patent No. 4,892,538; Aebischer et al. U.S. Patent No. 5,106,627; Hoffman et al. (1990) Expt. Neurobiol. 110:39-44; Jaeger et al. (1990) Prog. Brain Res. 82:41-46; and Aebischer et al. (1991) J. Biomech. Eng. 113:178-183), or can be co-extruded with a polymer which acts to form a polymeric coat about the viral packaging cells (Lim U.S. Patent No. 4,391,909; Sefton U.S. Patent No. 4,353,888; Sugamori et al. (1989) Trans. Am. Artif. Intern. Organs 35:791-799; Sefton et al. (1987) Biotechnol. Bioeng. 29:1135-1143; and Aebischer et al. (1991) Biomaterials 12:50-55). Again, manipulation of the polymer can be carried out to provide for optimal release of viral particles.

D. Factors Produced by Genetically-Modified Stem Cells

Yet another aspect of the invention pertains to the identification and ultimate preparation of any factors, particularly extracellular factors, which may produced by the *LH-2* cells and which serve as inductive molecules for the proliferation of hematopoietic cells. Autocrine and/or paracrine factor(s) which are produced by the *LH-2* expressing cells can be provided as a conditioned media, or they can be purified by standard protein purification procedures known in the art.

As described in Examples 3 and 4, the ectopic expression of a hematopoietic gene such as *LH-2* produces a cell which secretes one or more factors that are able to induce the renewable phenotype, e.g., at least in a paracrine fashion. While not wishing to be bound by any particular theory, it is nevertheless noted that the observations suggest that the factor found in conditioned media is a polypeptide which is either secreted as a soluble protein or which is an extracellular portion of a cell membrane protein that is released by cleavage or the like.

Various methods are employed to isolate and purify the inductive factor(s) from conditioned media. To examine the nature of the soluble factors in the conditioned media, the factors can be isolated by such art techniques as may include differential precipitation, molecular sieve chromatography, ion-exchange chromatography, isoelectric focusing, dialysis, gel electrophoresis and affinity and immunoaffinity chromatography. Fractions enriched in the proliferation activity can be identified using an assay based on, for

example, the ability of a particular fraction to cause expansion of a normal stem cell culture. Fractions enriched in such activity can be further purified by standard methods until, for example, a single band resulted upon SDS-PAGE. Micro-sequencing and standard cloning techniques can then be used to identify the gene encoding the inductive factor.

Where intended for administration to an animal or addition to a cell culture as an additive, the are a variety of different formulations of the inductive factor from which to chose. As appropriate, the inductive factor is provided in a pharmaceutically acceptable carrier, e.g., which should be substantially inert so as not to act with the active component. Suitable inert carriers include water, alcohol, polyethylene glycol, mineral oil or petroleum gel, propylene glycol and the like.

To prepare the pharmaceutical compositions of this invention, an effective amount of the factor as the active ingredient is combined in intimate admixture with a pharmaceutically acceptable carrier, which carrier may take a wide variety of forms depending on the form of preparation desired for administration. These pharmaceutical compositions are desirable in unitary dosage form suitable, particularly, for administration orally, rectally, percutaneously, or by parenteral injection. For example, in preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed such as, for example, water, glycols, oils, alcohols and the like in the case of oral liquid preparations such as suspensions, syrups, elixirs and solutions; or solid carriers such as starches, sugars, kaolin, lubricants, binders, disintegrating agents and the like in the case of powders, pills, capsules, and tablets. Because of their ease in administration, tablets and capsules represents the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. For parenteral compositions, the carrier will usually comprise sterile water, at least in large part, though other ingredients, for example, to aid solubility, may be included. Injectable solutions, for example, may be prepared in which the carrier comprises saline solution, glucose solution or a mixture of saline and glucose solution. Injectable suspensions may also be prepared in which case appropriate liquid carriers, suspending agents and the like may be employed. Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations. In the compositions suitable for percutaneous administration, the carrier optionally comprises a penetration enhancing agent and/or a suitable wetting agent, optionally combined with suitable additives of any nature in minor proportions, which additives do not introduce a significant deleterious effect on the skin.

It is especially advantageous to formulate the subject compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used in the specification and claims herein refers to physically discreate units suitable as unitary

dosages, each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. Examples of such dosage unit forms are tablets (including scored or coated tablets), capsules, pills, powders packets, wafers, injectable solutions or suspensions, teaspoonfuls, tablespoonfuls and the like, and segregated multiples thereof.

The pharmaceutical preparations of the present invention can be used, as stated above, for the many applications which can be considered cosmetic uses. Cosmetic compositions known in the art, preferably hypoallergic and pH controlled are especially preferred, and include toilet waters, packs, lotions, skin milks or milky lotions. The preparations may contain, besides the inductive factor, components usually employed in such preparations. Examples of such components are oils, fats, waxes, surfactants, humectants, thickening agents, antioxidants, viscosity stabilizers, chelating agents, buffers, preservatives, perfumes, dyestuffs, lower alkanols, and the like. If desired, further ingredients may be incorporated in the compositions, e.g. anti-inflammatory agents, antibacterials, antifungals, disinfectants, vitamins, sunscreens, antibiotics, or other anti-acne agents.

Examples of oils comprise fats and oils such as olive oil and hydrogenated oils; waxes such as beeswax and lanolin; hydrocarbons such as liquid paraffin, ceresin, and squalane; fatty acids such as stearic acid and oleic acid; alcohols such as cetyl alcohol, stearyl alcohol, lanolin alcohol, and hexadecanol; and esters such as isopropyl myristate, isopropyl palmitate and butyl stearate. As examples of surfactants there may be cited anionic surfactants such as sodium stearate, sodium cetylsulfate, polyoxyethylene laurylether phosphate, sodium N-acyl glutamate; cationic surfactants such as stearyldimethylbenzylammonium chloride and stearyltrimethylammonium chloride; ampholytic surfactants such as alkylaminoethylglycine hydrocloride solutions and lecithin; and nonionic surfactants such as glycerin monostearate, sorbitan monostearate, sucrose fatty acid esters, propylene glycol monostearate, polyoxyethylene oleylether, polyethylene glycol monostearate, polyoxyethylene sorbitan monopalmitate, polyoxyethylene coconut fatty acid monoethanolamide, polyoxypropylene glycol (e.g. the materials sold under the trademark "Pluronic"), polyoxyethylene castor oil, and polyoxyethylene lanolin. Examples of humectants include glycerin, 1,3-butylene glycol, and propylene glycol; examples of lower alcohols include ethanol and isopropanol; examples of thickening agents include xanthan gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, polyethylene glycol and sodium carboxymethyl cellulose; examples of antioxidants comprise butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, citric acid and ethoxyquin; examples of chelating agents include disodium edetate and ethanehydroxy diphosphate; examples of buffers comprise citric acid, sodium citrate, boric acid, borax, and disodium

hydrogen phosphate; and examples of preservatives are methyl parahydroxybenzoate, ethyl parahydroxybenzoate, dehydroacetic acid, salicylic acid and benzoic acid.

For preparing ointments, creams, toilet waters, skin milks, and the like, typically from 0.01 to 10% in particular from 0.1 to 5% and more in particular from 0.2 to 2.5% of the active ingredient, e.g., of the inductive factor, will be incorporated in the compositions. In ointments or creams, the carrier for example consists of 1 to 20%, in particular 5 to 15% of a humectant, 0.1 to 10% in particular from 0.5 to 5% of a thickener and water; or said carrier may consist of 70 to 99%, in particular 20 to 95% of a surfactant, and 0 to 20%, in particular 2.5 to 15% of a fat; or 80 to 99.9% in particular 90 to 99% of a thickener; or 5 to 15% of a surfactant, 2-15% of a humectant, 0 to 80% of an oil, very small (< 2%) amounts of preservative, coloring agent and/or perfume, and water. In a toilet water, the carrier for example consists of 2 to 10% of a lower alcohol, 0.1 to 10% or in particular 0.5 to 1% of a surfactant, 1 to 20%, in particular 3 to 7% of a humectant, 0 to 5% of a buffer, water and small amounts (< 2%) of preservative, dyestuff and/or perfume. In a skin milk, the carrier typically consists of 10-50% of oil, 1 to 10% of surfactant, 50-80% of water and 0 to 3% of preservative and/or perfume. In the aforementioned preparations, all % symbols refer to weight by weight percentage.

Particular compositions for use in the method of the present invention are those wherein the factor is formulated in liposome-containing compositions. Liposomes are artificial vesicles formed by amphiphatic molecules such as polar lipids, for example, phosphatidyl cholines, ethanolamines and serines, sphingomyelins, cardiolipins, plasmalogens, phosphatidic acids and cerebiosides. Liposomes are formed when suitable amphiphathic molecules are allowed to swell in water or aqueous solutions to form liquid crystals usually of multilayer structure comprised of many bilayers separated from each other by aqueous material (also referred to as coarse liposomes). Another type of liposome known to be consisting of a single bilayer encapsulating aqueous material is referred to as a unilamellar vesicle. If water-soluble materials are included in the aqueous phase during the swelling of the lipids they become entrapped in the aqueous layer between the lipid bilayers.

Water-soluble active ingredients such as, for example, various salt forms of a inductive factor, are encapsulated in the aqueous spaces between the molecular layers. The encapsulation of these proteins can be achieved by a number of methods. The method most commonly used involves casting a thin film of phospholipid onto the walls of a flask by evaporation from an organic solvent. When this film is dispersed in a suitable aqueous medium, multilamellar liposomes are formed. Upon suitable sonication, the coarse liposomes form smaller similarly closed vesicles.

Water-soluble active ingredients are usually incorporated by dispersing the cast film with an aqueous solution of the compound. The unencapsulated compound is then removed by centrifugation, chromatography, dialysis or other art-known suitable procedures. The lipid-soluble active ingredient is usually incorporated by dissolving it in the organic solvent with the phospholipid prior to casting the film. If the solubility of the material in the lipid phase is not exceeded or the amount present is not in excess of that which can be bound to the lipid, liposomes prepared by the above method usually contain most of the material bound in the lipid bilayers; separation of the liposomes from unencapsulated material is not required.

A particularly convenient method for preparing liposome formulated forms of the factor is the method described in EP-A-253,619, incorporated herein by reference. In this method, single bilayered liposomes containing encapsulated active ingredients are prepared by dissolving the lipid component in an organic medium, injecting the organic solution of the lipid component under pressure into an aqueous component while simultaneously mixing the organic and aqueous components with a high speed homogenizer or mixing means, whereupon the liposomes are formed spontaneously.

The single bilayered liposomes containing the encapsulated factor can be employed directly or they can be employed in a suitable pharmaceutically acceptable carrier for localized administration. The viscosity of the liposomes can be increased by the addition of one or more suitable thickening agents such as, for example xanthan gum, hydroxypropyl cellulose, hydroxypropyl methylcellulose and mixtures thereof. The aqueous component may consist of water alone or it may contain electrolytes, buffered systems and other ingredients, such as, for example, preservatives. Suitable electrolytes which can be employed include metal salts such as alkali metal and alkaline earth metal salts. The preferred metal salts are calcium chloride, sodium chloride and potassium chloride. The concentration of the electrolyte may vary from zero to 260 mM, preferably from 5 mM to 160 mM. The aqueous component is placed in a suitable vessel which can be adapted to effect homogenization by effecting great turbulence during the injection of the organic component. Homogenization of the two components can be accomplished within the vessel, or, alternatively, the aqueous and organic components may be injected separately into a mixing means which is located outside the vessel. In the latter case, the liposomes are formed in the mixing means and then transferred to another vessel for collection purpose.

The organic component consists of a suitable non-toxic, pharmaceutically acceptable solvent such as, for example ethanol, glycerol, propylene glycol and polyethylene glycol, and a suitable phospholipid which is soluble in the solvent. Suitable phospholipids which can be employed include lecithin, phosphatidylcholine,

phosphatydylserine, phosphatidylethanol-amine, phosphatidylinositol, lysophosphatidyl-choline and phospha-tidyl glycerol, for example. Other lipophilic additives may be employed in order to selectively modify the characteristics of the liposomes. Examples of such other additives include stearylamine, phosphatidic acid, tocopherol, cholesterol and lanolin extracts.

In addition, other ingredients which can prevent oxidation of the phospholipids may be added to the organic component. Examples of such other ingredients include tocopherol, butylated hydroxyanisole, butylated hydroxytoluene, ascorbyl palmitate and ascorbyl oleate. Preservatives such a benzoic acid, methyl paraben and propyl paraben may also be added.

In another aspect, the present invention facilitates the cloning of genes involved in the regeneration paradigm accompanying expression of *LH-2*. By "cloning" it is meant the isolation of a nucleic acid sequence encoding a factor of interest, e.g., from RNA or DNA sources. For instance, the subject cells can be used for the identification, isolation, and study of genes and gene products that provide an inductive signal for proliferation of hematopoietic stems cells. To illustrate, if new transcription is required for the production of one or more inductive proteins, a subtractive cDNA library prepared after hybridizing mRNA from control target cells (no ectopic *LH-2* expression) to mRNA from experimental target cells (expressing recombinant *LH-2*) can be used to isolate genes that are turned on or turned off by the expression of the *LH-2* transcription factor. This type of subtractive approach has been used successfully to isolate a variety of new genes (see, for example, Wang et al. (1991) *PNAS. USA* 88:11505-11509).

A similar approach involves the use of "differential display". See, for example, Liang et al. (1992) *Cancer Res* 52:6966-6998; Liang et al. (1992) *Science* 257:967-971; and Liang et al. (1993) *Nuc Acid Res* 21:3269-3275. The strategy of the method is to use PCR to amplify partial cDNAs that have been made by reverse transcription of RNAs isolated from the two cells of interest, in the case a hematopoietic stem cell which is engineered to express *LH-2* and one which does not. The PCR products are then separated in adjacent lanes on a denaturing polyacrylamide gel, and products that are made in one tissue but not in the other are cut directly from the gel, subcloned and sequenced.

Since it is only possible to get good resolution of ~100 PCR products on a single gel, the PCR primers are selected so that only subsets of the RNAs are represented in each PCR reaction. This is accomplished by carrying out the reverse transcription using an oligo-dT primer that consists of 12 T's and two additional 3' bases that confer specificity. This oligo-dT primer is also used in the PCR reactions along with a second primer of arbitrary sequence, which is expected to anneal to different cDNAs at different

distances away from the polyA$^+$ tail. By varying the sequence of this second primer and the sequence of the last base in the oligo-dT primer it is possible to use this procedure to amplify most of the polyA$^+$-containing mRNAs that are believed to be expressed in a typical cell at any one time.

The differential display method can be used to identify genes whose transcription is stimulated by expression of the heterologous transcription factor. RNA will be isolated from cells that have been recombinantly modified to express *LH-2*, and from identical cells which do not express *LH-2*. The RNA from each cell-type will then be reverse transcribed in four separate reactions using oligo-dT primers with dG, dA, dT or dC at the 3' terminus. The resulting cDNAs will be amplified in the presence of [^{35}S]dATP using a set of primers consisting of one of the four oligo-dT primers and one of 20 different commercially available primers of arbitrary sequence. Thus, for each tissue there will be 80 different PCR reactions, each involving the use of a different set of primers. The PCR products obtained using each set of primers will then be resolved on a denaturing polyacrylamide gel, with the PCR products resulting from the amplification of cDNA from the control target tissue run side-by-side with the PCR products resulting from the amplification of cDNA from the experimental target tissue. The PCR products will be visualized by autoradiography, and any products that are present in one lane but not the other will be cut out of the gel, subcloned and sequenced. Presumably these products represent the amplification of genes that are transcribed as a consequence of ectopic expression of the transcription factor.

According to another embodiment of the present invention, there is provided a therapeutic preparation of an expression construct for ectopically expressing the inductive factor, or derivatives thereof (such truncated forms, fusion proteins, point mutants, etc.). The expression vector can be provided in the form of a viral vector, such as a retrovirus, adenovirus, adeno-associated virus, herpes virus, or the like *(supra)*, or as a non-viral system such as liposomes, poly-lysine conjugates, artificial viral envelopes and the like *(supra)*. The expression vector can, as appropriate, be provided in a pharmaceutical carrier as described above.

VII. Genetically Modified Feeder Layers

An alternate embodiment of the subject invention is provided by the observation that one or more factors produced by cells engineered to ectopically express *LH-2* may serve as autocrine/paracrine factors. Accordingly, as certain of the data provided in the appended examples indicates, cells which are ectopically expressing *LH-2* may, in a mixed cell culture, be capable of producing factors which induce the renewal phenotype in unmodified cells.

Accordingly, another aspect of the present invention provides a feeder cell, preferably in the form of a feeder cell layer, which is modified to ectopically express *LH-2* (or some other appropriate hematopoietic gene), and normal embryonic or hematopoietic stem cells are cultured with that feeder cell, preferably in the presence of steel factor. Using techniques known in the art, the expanded stem cell population can be isolated from the feeder cells. A benefit to such embodiments the ability to avoid the need to genetically engineer the stem cells.

VIII. Lineage-restricted Stem Cells

Another aspect of the present invention pertains to stem cells which have been modified by a loss-of-function mutation which restricts the hematopoietic lineages accessible to the cell when reimplanted. While the lineages created by differentiation can be more closely controlled in the case of *ex vivo* cultures, it will be appreciated that the extracellular signals encountered by hematopoietic stem *in vivo* may include signals ultimately producing all kinds of differentiated hematopoietic cells. Loss-of-function mutations, e.g., by such methods as homologous recombination, antisense or dominant negative mutants, can provide a means for limiting the ultimate differentiated cell types occurring *in vivo*. Thus, for example, loss-of-function mutations to GATA-3 and PU.1 should result in stem cells which, when implanted, have a reduced capacity to differentiate into B- or T-cells, e.g., will be more likely to produce myeloid lineages.

Homologous recombination

One method for causing a loss-of-function mutation to a gene in ES cells is targeted gene replacement, e.g., by homologous recombination, with a targeting gene construct. For instance, such constructs can be derived to include: (i) at least one recombination region having a sequence that is substantially identical to or substantially complementary to the targeted gene sequence, or sequences flanking that gene, present in the host stem cell, and (ii) a replacement region which becomes integrated into the host cell's genome. By homologous recombination with the genomic sequence, the host cell's gene is disrupted by the targeting construct. For example, integration can occur in the transcriptional regulatory region and cause loss of transcription of the gene. In another embodiment, the disrupting construct can be integrated in the coding sequence so as to disrupt the expression of a functional gene product, e.g., by frameshift, premature stop codon or the like.

The disruption construct can be introduced into cultured ES cells by standard methods, e.g., electroporation, microinjection, and calcium phosphate treatment. A

preferred method of insertion is electroporation. Each gene construct to be inserted into the cell must first be in the linear form. Therefore, if the gene construct has been inserted into a vector, linearization is accomplished by digesting the DNA with a suitable restriction endonuclease selected to cut only within the vector sequence and not within the gene construct sequence.

For targeted insertion, the gene construct is added to the ES cells under appropriate conditions for the insertion method chosen, as is known to the skilled artisan. Where more than one construct is to be introduced into the ES cell, each gene construct can be introduced simultaneously or one at a time.

If the ES cells are to be electroporated, the ES cells and gene construct DNA are exposed to an electric pulse using an electroporation machine and following the manufacturer's guidelines for use. After electroporation, the ES cells are typically allowed to recover under suitable incubation conditions. The cells are then screened for the presence of the gene construct .

Screening of the transfected cells can be accomplished using a variety of methods. Where the construct includes a marker gene, e.g., an antibiotic resistance gene, the ES cells may be cultured in the presence of an otherwise lethal concentration of antibiotic. Those ES cells that survive have presumably integrated the gene construct. If the marker gene is other than an antibiotic resistance gene, a Southern blot of the ES cell genomic DNA can be probed with a sequence of DNA designed to hybridize only to the marker sequence Alternatively, PCR can be used. Finally, if the marker gene is a gene that encodes an enzyme whose activity can be detected (e.g., β-galactosidase), the enzyme substrate can be added to the cells under suitable conditions, and the enzymatic activity can be analyzed. One skilled in the art will be familiar with other useful markers and the means for detecting their presence in a given cell. All such markers are contemplated as being included within the scope of the teaching of this invention.

The gene construct may integrate into several locations in the ES cell genome, and may integrate into a different location in each ES cell's genome due to the occurrence of random insertion events. Typically, less than about 1-5 percent of the ES cells that take up the gene construct will actually integrate the gene construct in the desired location. To identify those ES cells with proper integration of the gene construct, total DNA can be extracted from the ES cells using standard methods. The DNA can then be probed on a Southern blot with a probe or probes designed to hybridize in a specific pattern to genomic DNA digested with particular restriction enzyme(s). Alternatively, or additionally, the genomic DNA can be amplified by PCR with probes specifically designed to amplify DNA fragments of a particular size and sequence (i.e., only those cells containing the gene construct in the proper position will generate DNA fragments of the proper size).

In one embodiment, gene targeting, which is a method of using homologous recombination to modify an animal's genome, can be used to introduce changes into cultured embryonic stem cells. By targeting an hematopoietic gene of interest in ES cells, these changes can be introduced into the germlines of animals to generate chimeras. The gene targeting procedure is accomplished by introducing into tissue culture cells a DNA targeting construct that includes a segment homologous to a target hematopoietic locus, and which also includes an intended sequence modification to the hematopoietic genomic sequence (e.g., insertion, deletion, point mutation). The treated cells are then screened for accurate targeting to identify and isolate those which have been properly targeted.

Gene targeting in embryonic stem cells is in fact a scheme contemplated by the present invention as a means for disrupting an hematopoietic gene function through the use of a targeting transgene construct designed to undergo homologous recombination with one or more genomic sequences from the hematopoietic gene. The targeting construct can be arranged so that, upon recombination with an element of an hematopoietic gene, a positive selection marker is inserted into (or replaces) coding sequences of the targeted hematopoietic gene. The inserted sequence functionally disrupts the hematopoietic gene, while also providing a positive selection trait.

Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example 1: Differentiation of ES cells into hematopoietic cells

Most of the experiments described below have been carried out using the 129 derived ES cell line CCE (e.g., ES cells of the 129 strain mouse). However, other ES cell lines, such as J1, worked equally well under the same conditions. The ES cells were routinely cultured without feeder cells in the presence of leukemia inhibitory factor (LIF). The standard differentiation assay was as follows: ES cells were differentiated for 6 days to form embryoid bodies (EBs) as described in the art with some modifications. EBs were harvested and single cell suspensions were prepared. The single cell suspension was assayed for precursor content in colony forming cell-culture (CFC-c) assay as described in Keller et al., *supra*. This procedure will be hereinafter referred to as the precursor assays. The hematopoietic factors used in the precursor assay were primarily erythropoietin (epo) and steel factor (SF). At this stage of EB development, the combination of these factors

promotes the differentiation of precursors for the primitive and the definitive erythroid lineages, and to a lesser extent the megakaryocytic lineage.

In Figure 2, the typical morphology of the two types of erythroid colony can be seen. The small red and compact colony consists of primitive erythroid cells, i.e. large nucleated hemoglobinized cells expressing the fetal type of globins. This type of colony is already present 4-5 days after replating. The red large and irregular shaped colony consists of definitive erythroid cells, i.e. small anucleated hemoglobinized cells expressing the adult type of globins. This type of colonies does not appear until day 7-9 after replating. Some of the latter type of colony also contain megakaryocytes as shown by May-Grunwald Giemsa staining of cells derived from individual colonies (data not shown). In Figures 3 and 4, the frequencies of precursors for the respective lineage in experiments using different ES cell lines are displayed. Typically, at day 6 of differentiation the frequency of precursors for the primitive erythroid lineage is approximately 1/100 EB derived cells, and the frequency of precursors for the definitive erythroid lineage is 5-30/10^5 cells. Typically, 10^5 EB-derived cells were plated per dish. Thus, the number of precursors per dish is equivalent to the number of hematopoietic precursors present in 10 embryos at embryonic day 8.

Example 2: Analysis of the role of the LH-2 gene product in the development of the HS utilizing the ES cell system

In order to be able to study a putative function of the Lhx gene *LH-2* in the development and expansion of the hematopoietic system (HS), a retroviral system was established. The retroviral vector was modified for efficient expression of genes in ES cells, and is hereinafter referred to as the Murine Stem Cell Virus (MSCV) vector. The *LH-2* gene was cloned into the multiple cloning site of the MSCV vector. The structures of the vector only (MSCV) and the *LH-2* containing vector (MSCV-*LH-2*) are shown in Figure 5. Virions were produced and harvested by transfecting these two vectors into the virus producing cell line BOSC-23 as described. Since the MSCV vector contains the neomycin phosphotransferase (neo) gene, infected CCE ES cells were selected for G418 resistance. Both ES cells from the bulk infected population (i.e. non-subcloned) and subclones were analyzed. No major morphological, or growth rate differences were observed between the MSCV-*LH-2* infected, MSCV infected, or non-infected ES cells, indicating that *LH-2* expression did not alter the undifferentiated state of the ES cells. These different populations of infected ES cells were subjected to the precursor cell differentiation assay described above. No difference in the frequency of precursors for primitive erythroid cells was detected when comparing the MSCV and the MSCV-*LH-2* infected bulk populations (Figure 3a). An increase in the frequency of precursor for the

definitive erythroid cells was, however, detected in the MSCV-*LH-2* bulk population (53/2x105 cells) as compared to the MSCV bulk population (31/2x105 cells) (see Figure 3b).

An additional very interesting difference was that in the clonal assays of the EBs derived from the MSCV-*LH-2* transfected ES cells, a novel type of colony appeared, not present in any EBs derived from MSCV transfected or non-transfected ES cells. The frequency of this type of colony in the MSCV-*LH-2* bulk population was approximately 10-20 times less than the frequency of definitive erythroid colonies. In one of four subclones tested of the MSCV-*LH-2* group, this novel type of colony appeared in an equal frequency as that for the definitive erythroid colonies, and it grew bigger as compared to the MSCV-*LH-2* bulk group. In contrast to all other colonies where a vast majority of cells within a colony hemoglobinize (turned red) within 7-8 days, this novel type of colony never completely hemoglobinize, but red "spots" appear within the colony. A typical large non-red colony with these red "spots" is shown in Figure 6. This figure illustrates the typical appearance of these colonies after 1.5-2 weeks after replating. The majority of these cells show an "immature" phenotype, i.e. no clear morphology but blast like cells with a large round nuclei and a small rim of cytoplasm, as detected by May-Grunwald Giemsa stain. In addition to these immature cells, megakaryocytes and anucleated erythrocytes were present. These data suggest that the majority of the cells in this colony are immature cells that have expanded without concomitant commitment to any particular hematopoietic lineage. To test this hypothesis, a number of individual colonies were picked and replated in precursor assays containing interleukin-3 (IL-3) in addition to EPO and SF. This assay should expand the number of other types of precursors. As determined by colony morphology and May-Grunwald Giemsa staining of cells derived from individual colonies in this secondary precursor assay, colonies of immature cells reappeared as well as colonies containing pure erythroid cells, erythroid/megakaryocytes, erythroid/macrophages, and erythrocyte/megakaryocyte/ neutrophil/macrophages (data not shown). Furthermore, cells derived from this type of colony did not grow when assayed in precursor assays lacking growth factors. In order to address more specifically the growth factor dependency of these cells, cell cultures were transferred into liquid cultures containing either EPO, SF, or IL-3, and SF in combination with IL-3. SF was the only factor able to promote the growth of these cells. No significant growth was observed in the presence of IL-3 (data not shown). More importantly, the withdrawal of SF and addition of EPO promoted immediate and massive terminal differentiation of erythroid precursors (data not shown).

These data shows that this experimental protocol has generated a multilineage precursor capable of self-renewal while maintaining the ability to differentiate into a variety of mature hematopoietic cell types. This type of cell will hereinafter be referred to

as the putative hematopoietic progenitor cells (HPCs). This is a very important finding because the study of the equivalent cell *in vivo* is severely hampered by its extremely low abundance. Thus, a pure and homogenous population of these cells are almost impossible to obtain *in vivo* with the techniques available today. This has led to indirect characterization of these cells. The establishment of a putative HPCs makes it possible to directly characterize progenitor cell properties at the cellular, molecular and biochemical level. Furthermore, the transfection of human (or mouse) *LH-2* into purified human hematopoietic stem/progenitor cells from, for example human cord blood, could make it possible to establish equivalent cells from humans. Thus, making similar characterization of human stem cells feasible.

Preliminary reports on the phenotype of *LH-2* knock out mice reveal interesting cues to a possible mechanism of *LH-2* mediated proliferation of hematopoietic stem cells. The homozygous *LH-2* knock out mice die at 17 days of gestation presumably due to a reduction in erythroid precursors that leads to severe anemia. Furthermore, in mice chimeric for wild type cells and homozygous for *LH-2* mutant cells, homozygous mutant cells can form cells of the erythroid and lymphoid lineages. This result strongly suggests a cell non-autonomous defect in which the *LH-2* homeodomain protein regulates the expression of a factor that in concert with steel factor can expand immature hematopoietic cells without significant differentiation.

The possible clinical applications of these findings include: i) the isolation of limited numbers of hematopoietic precursors from patients and their *in vitro* expansion to create an unlimited supply of cells for use in transplantation paradigms. This could have applications in the treatment of illnesses requiring bone marrow transplantation, such as all cancer treatments involving radio- or chemotherapy, ii) a possible cure for auto immune diseases by replacing pathogenic lymphocytes iii) a possible cure for different kinds of inherited or acquired anemias, iv) AIDS if the possibility exists that the HIV infected compartment can be replaced with healthy cells by expanding a small number of the patients own non-infected stem cells.

Example 3: Cells expressing LH-2 gene product can induce renewal phenotype in other co-cultured cells

As described above, forced expression of *LH-2* in hematopoietic cells expand immature cells in the presence of Steel factor, a factor crucial for the establishment of fetal liver hematopoiesis. In long term bone marrow cultures, Steel factor only promotes differentiation, not self-renewal of immature cells. This data, together with the fact that *LH-2* is expressed in the fetal liver, would implicate *LH-2* expression in the expansion of the HPC in the fetal liver. There are two possible mechanisms, cell extrinsic or cell

intrinsic. Regarding the former, the fact that LH2-/- cells in LH2+/+<->LH2-/- chimeric mice can contribute to cells of the hematopoietic system (a cell non-autonomous defect), suggests that *LH-2* expression promotes the expression of a cell-cell signaling molecule(s), soluble and/or membrane bound.

To test this possibility, mitotically inactivated wild-type or *LH-2* expressing ES cells were mixed with wild-type cells in a precursor assay, with or without epo/steel factor. Without factors, no hematopoietic colonies of adult type appeared, indicating that the *LH-2* expressing cells did not produce any of the known factors that can stimulate colony formation, e.g., Steel factor, IL-3, CSFs, etc. When epo/Steel factor was added to the mixed cell cultures, the plating efficiency of the wild-type cells increased by 100% for the colonies of the adult eryhroid cells when mitotically inactive *LH-2* expressing cells were used to co-culture (compared to co-cultures with mitotically inactivated wild-type cells). This indicates that a factor produced by the ectopic expression of *LH-2* may be capable of inducing the formation of the renewal phenotype stem cell in mixed cultures. Moreover, since a large proportion of the additional colonies were adult erythroid colonies, this data indicates that an earlier precursor, e.g., not yet epo/Steel factor responsive, was present in the wild-type ES cells.

Example 4: Conditioned media can induce renewal phenotype

To further identification of a stem cell inducing factor(s) which may be produced by the ectopic expression of *LH-2*, conditioned media samples were further tested for their ability to generate the self renewable phenotype in a methylcellulose colony assay. Briefly, it was first observed in liquid cultures that conditioned medium from the subject *LH-2* expressing cells can support the proliferation and/or survival of dilute populations of hematopoietic stem cells. In liquid culture, it was observed that when hematopoietic stem cells are diluted to less than 1000 cells/ml, they die despite supplementation with steel factor. Addition of conditioned medium from the *LH-2* expressing cells described herein can rescue the cultured cells under these conditions --supporting the notion that ectopic expression of *LH-2* can induce the expression/secretion of one or more soluble factors which can induce a renewable phenotype in cultured hematopoietic cells.

To provide a more quantitative means for detecting the presence of one or more factors in the *LH-2* induced media which are responsible for rescue of the diluted cells, a methylcellulose-based assay was adapted from the art (see, for example, Eaves et al., (1978) *Blood* 52:1196-1210). In the this assay, HPC's are plated into culture dishes containing 1% methylcellulose in Iscove's Modified Dulbecco's Medium (IMDM), 10% fetal calf serum (FCS), steel factor (100 ng/ml), with or without added conditioned medium. Figure 7 shows that at 10^6 cells/ml, HPC cells give rise to colonies independent

of the addition of conditioned medium. However, at $5x10^4$ cells/ml, colony formation is dependent upon addition of conditioned medium (see Figure 8), confirming that one or more secreted factors are produced by ectopic expression of *LH-2*, which factors can induce a renewable phenotype in cultured hematopoietic cells.

This assay is more sensitive relative to the liquid assay, and the colonies can be enumerated and examined for size, and removed from the dish for further study. Figure 9 shows the results of several assays in which varying numbers of HPC cells were plated into cultures +/- 30% conditioned medium. The subject methylcellulose colony assay can be used to fractionate and otherwise purify the *LH-2*-induced factor(s) which give rise to the renewable phenotype.

All of the above-cited references and publications are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1888 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 489..1700

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCTTGAAATC GAATTCGGGA TTCGGGGGGG ACGCACCAGG GAGGGAGGGG TCCAGGCAGC
60

TGGGCCGCCG CGGACACCTA GCGGCTTCAG GGTGAACCCC GACCGCAGCC GTCGCCGCCT
120

CGGGCAGAGT TTGCGCCCTT GCTTTGCGCC CCGCTGCGAA GCCGGGCGGG CGATCGGCGC
180

GTGAAAGCGC CGCGCGGGCG ACCTCTGTCC TAGTCTCCTG CTCCCCCCGC CCCGCTTGTC
240

CCGTGCCCTT GTGACCCAGG CTTTGGCGCC GTCGCCAGGC CCCGCAATGT AGCTGCCCCT
300

GCGCCTCGGC GGAGGCTCCT GCCCCGCGAG CGCCCGGGGC CCGGAGCCGG CCTGGGGGCT
360

CAGCCGAGCT CGGGCGGGGC CGGGGCGCGG TGGCGATGCA CCGGGCCGTT AGCGCCAGGA
420

GCCAGGCAGC TGAGGCGGGG GGCAAGCCTC CCTCGGAGAG CCGCGCCCCC GGCCCGCGTC
480

CCGCCGCG ATG CTG TTC CAC AGT CTG TCG GGC CCC GAG GTG CAC GGG GTC
530
Met Leu Phe His Ser Leu Ser Gly Pro Glu Val His Gly Val
1 5 10

ATC GAC GAG ATG GAC CGC AGG CAA GAG CGA GGC TCC CGC ATC AGC TCC
578
Ile Asp Glu Met Asp Arg Arg Gln Glu Arg Gly Ser Arg Ile Ser Ser
15 20 25 30

GCC ATC GAC CGC GGC GAC ACC GAG ACG ACC ATG CCG TCC ATC AGC AGT
626
Ala Ile Asp Arg Gly Asp Thr Glu Thr Thr Met Pro Ser Ile Ser Ser

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                    35                  40                  45

GAC CGC GCC GCC CTT TGT GGC GGC TGT GGC GGC AAG ATC TCG GAC CGC
674
Asp Arg Ala Ala Leu Cys Gly Gly Cys Gly Gly Lys Ile Ser Asp Arg
          50                  55                  60

TAC TAC CTG CTG GCG GTG GAC AAG CAG TGG CAC ATG CGC TGC CTC AAG
722
Tyr Tyr Leu Leu Ala Val Asp Lys Gln Trp His Met Arg Cys Leu Lys
      65                  70                  75

TGC TGC GAG TGC AAG CTC AAC CTG GAG TCG GAG CTC ACC TGT TTC AGC
770
Cys Cys Glu Cys Lys Leu Asn Leu Glu Ser Glu Leu Thr Cys Phe Ser
  80                  85                  90

AAG GAC GGT AGC ATC TAC TGC AAG GAA GAC TAC TAC CGG CGC TTC TCT
818
Lys Asp Gly Ser Ile Tyr Cys Lys Glu Asp Tyr Tyr Arg Arg Phe Ser
 95                  100                 105                 110

GTG CAG CGC TGC GCC CGC TGC CAC CTG GGC ATC TCG GCC TCG GAG ATG
866
Val Gln Arg Cys Ala Arg Cys His Leu Gly Ile Ser Ala Ser Glu Met
                115                 120                 125

GTG ATG CGC GCT CGG GAC TTG GTT TAT CAC CTC AAC TGC TTC ACG TGC
914
Val Met Arg Ala Arg Asp Leu Val Tyr His Leu Asn Cys Phe Thr Cys
            130                 135                 140

ACC ACG TGT AAC AAG ATG CTG ACC ACG GGC GAC CAC TTC GGC ATG AAG
962
Thr Thr Cys Asn Lys Met Leu Thr Thr Gly Asp His Phe Gly Met Lys
        145                 150                 155

GAC AGC CTG GTC TAC TGC CGC TTG CAC TTC GAG GCG CTG CTG CAG GGC
1010
Asp Ser Leu Val Tyr Cys Arg Leu His Phe Glu Ala Leu Leu Gln Gly
    160                 165                 170

GAG TAC CCC GCA CAC TTC AAC CAT GCC GAC GTG CAG GCG GCG CGT GCA
1058
Glu Tyr Pro Ala His Phe Asn His Ala Asp Val Gln Ala Ala Arg Ala
175                 180                 185                 190

CGC GCG GCG GCC AAG AGC GCG GGG CTG GGC GCA GCA GGG GCC AAC CCT
1106
Arg Ala Ala Ala Lys Ser Ala Gly Leu Gly Ala Ala Gly Ala Asn Pro
                195                 200                 205

CTG GGT CTT CCC TAC TAC AAT GGC GTG GGC ACT GTG CAG AAG GGG CGG
1154
Leu Gly Leu Pro Tyr Tyr Asn Gly Val Gly Thr Val Gln Lys Gly Arg
            210                 215                 220
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CCG AGG AAA CGT AAG AGT CCG GGC CCC GGT GCG GAT CTG GCG GCC TAC
1202
Pro Arg Lys Arg Lys Ser Pro Gly Pro Gly Ala Asp Leu Ala Ala Tyr
        225                 230                 235

ACA CGT GCG CTA AGC TGC AAC GAA AAC GAC GCA GAG CAC CTG GAC CGT
1250
Thr Arg Ala Leu Ser Cys Asn Glu Asn Asp Ala Glu His Leu Asp Arg
    240                 245                 250

GAC CAG CCA TAC CCC AGC AGC CAG AAG ACC AAG CGC ATG CGC ACG TCC
1298
Asp Gln Pro Tyr Pro Ser Ser Gln Lys Thr Lys Arg Met Arg Thr Ser
255                 260                 265                 270

TTC AAG CAC CAC CAG CTT CGG ACC ATG AAG TCT TAC TTT GCC ATT AAC
1346
Phe Lys His His Gln Leu Arg Thr Met Lys Ser Tyr Phe Ala Ile Asn
                275                 280                 285

CAC AAT CCC GAT GCC AAG GAC TTG AAG CAG CTC GCG CAA AAG ACG GGC
1394
His Asn Pro Asp Ala Lys Asp Leu Lys Gln Leu Ala Gln Lys Thr Gly
            290                 295                 300

CTC ACC AAG CGG GTC CTC CAG GTC TGG TTC CAG AAC GCC CGA GCC AAG
1442
Leu Thr Lys Arg Val Leu Gln Val Trp Phe Gln Asn Ala Arg Ala Lys
        305                 310                 315

TTC AGG CGC AAC CTC TTA CGG CAG GAA AAC ACG GGC GTG GAC AAG TCG
1490
Phe Arg Arg Asn Leu Leu Arg Gln Glu Asn Thr Gly Val Asp Lys Ser
    320                 325                 330

ACA GAT GCG GCG CTG CAG ACA GGG ACG CCA TCG GGC CCG GCC TCG GAG
1538
Thr Asp Ala Ala Leu Gln Thr Gly Thr Pro Ser Gly Pro Ala Ser Glu
335                 340                 345                 350

CTC TCC AAC GCC TCG CTC AGC CCC TCC AGC ACG CCC ACC ACC CTG ACA
1586
Leu Ser Asn Ala Ser Leu Ser Pro Ser Ser Thr Pro Thr Thr Leu Thr
                355                 360                 365

GAC TTG ACT AGC CCC ACC CTG CCA ACT GTG ACG TCC GTC TTA ACT TCT
1634
Asp Leu Thr Ser Pro Thr Leu Pro Thr Val Thr Ser Val Leu Thr Ser
            370                 375                 380

GTG CCT GGC AAC CTG GAG GGC CAT GAG CCT CAC AGC CCC TCA CAA ACG
1682
Val Pro Gly Asn Leu Glu Gly His Glu Pro His Ser Pro Ser Gln Thr
        385                 390                 395

ACT CTT ACC AAC CTT TTC TAATGACTCG CAACCCCCTC ACCCCACAAT
1730
Thr Leu Thr Asn Leu Phe
```

400

TTCTTTAAAA AAGAAATTAT CTTTAGTTTG AATTCCAAGT GTATTTTAAA ATAGAGGCTT
1790

TGAGCAACTA ACTAACCACA TTTTAGGATC TCGCCTGGAA ACAGAGGTAA AAAAAAGAAG
1850

TGTGCGCCCG GCTAATGCAG CGGTGTGGAC CGGAATTC
1888

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 404 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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1 5 10 15

Glu Met Asp Arg Arg Gln Glu Arg Gly Ser Arg Ile Ser Ser Ala Ile
20 25 30

Asp Arg Gly Asp Thr Glu Thr Thr Met Pro Ser Ile Ser Ser Asp Arg
35 40 45

Ala Ala Leu Cys Gly Gly Cys Gly Gly Lys Ile Ser Asp Arg Tyr Tyr
50 55 60

Leu Leu Ala Val Asp Lys Gln Trp His Met Arg Cys Leu Lys Cys Cys
65 70 75 80

Glu Cys Lys Leu Asn Leu Glu Ser Glu Leu Thr Cys Phe Ser Lys Asp
85 90 95

Gly Ser Ile Tyr Cys Lys Glu Asp Tyr Tyr Arg Arg Phe Ser Val Gln
100 105 110

Arg Cys Ala Arg Cys His Leu Gly Ile Ser Ala Ser Glu Met Val Met
115 120 125

Arg Ala Arg Asp Leu Val Tyr His Leu Asn Cys Phe Thr Cys Thr Thr
130 135 140

Cys Asn Lys Met Leu Thr Thr Gly Asp His Phe Gly Met Lys Asp Ser
145 150 155 160

Leu Val Tyr Cys Arg Leu His Phe Glu Ala Leu Leu Gln Gly Glu Tyr
165 170 175

Pro Ala His Phe Asn His Ala Asp Val Gln Ala Ala Arg Ala Arg Ala
180 185 190

Ala Ala Lys Ser Ala Gly Leu Gly Ala Ala Gly Ala Asn Pro Leu Gly
195 200 205

Leu Pro Tyr Tyr Asn Gly Val Gly Thr Val Gln Lys Gly Arg Pro Arg
210 215 220

Lys Arg Lys Ser Pro Gly Pro Gly Ala Asp Leu Ala Ala Tyr Thr Arg
225 230 235 240

Ala Leu Ser Cys Asn Glu Asn Asp Ala Glu His Leu Asp Arg Asp Gln
245 250 255

Pro Tyr Pro Ser Ser Gln Lys Thr Lys Arg Met Arg Thr Ser Phe Lys
260 265 270

His His Gln Leu Arg Thr Met Lys Ser Tyr Phe Ala Ile Asn His Asn
275 280 285

Pro Asp Ala Lys Asp Leu Lys Gln Leu Ala Gln Lys Thr Gly Leu Thr
290 295 300

Lys Arg Val Leu Gln Val Trp Phe Gln Asn Ala Arg Ala Lys Phe Arg
305 310 315 320

Arg Asn Leu Leu Arg Gln Glu Asn Thr Gly Val Asp Lys Ser Thr Asp
325 330 335

Ala Ala Leu Gln Thr Gly Thr Pro Ser Gly Pro Ala Ser Glu Leu Ser
340 345 350

Asn Ala Ser Leu Ser Pro Ser Ser Thr Pro Thr Thr Leu Thr Asp Leu
355 360 365

Thr Ser Pro Thr Leu Pro Thr Val Thr Ser Val Leu Thr Ser Val Pro
370 375 380

Gly Asn Leu Glu Gly His Glu Pro His Ser Pro Ser Gln Thr Thr Leu
385 390 395 400

Thr Asn Leu Phe

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1887 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 489..1757

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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60

TGGGCCGCCG CGGACACCTA GCGGCTTCAG GGTGAACCCC GACCGCAGCC GTCGCCGCCT
120

CGGGCAGAGT TTGCGCCCTT GCTTTGCGCC CCGCTGCGAA GCCGGGCGGG CGATCGGCGC
180

GTGAAAGCGC CGCGCGGGCG ACCTCTGTCC TAGTCTCCTG CTCCCCCCGC CCCGCTTGTC
240

CCGTGCCCTT GTGACCCAGG CTTTGGCGCC GTCGCCAGGC CCCGCAATGT AGCTGCCCCT
300

GCGCCTCGGC GGAGGCTCCT GCCCCGCGAG CGCCCGGGGC CCGGAGCCGG CCTGGGGGCT
360

CAGCCGAGCT CGGGCGGGGC CGGGGCGCGG TGGCGATGCA CCGGGCCGTT AGCGCCAGGA
420

GCCAGGCAGC TGAGGCGGGG GGCAAGCCTC CCTCGGAGAG CCGCGCCCCC GGCCCGCGTC
480

CCGCCGCG ATG CTG TTC CAC AGT CTG TCG GGC CCC GAG GTG CAC GGG GTC
530
Met Leu Phe His Ser Leu Ser Gly Pro Glu Val His Gly Val
1 5 10

ATC GAC GAG ATG GAC CGC AGG CAA GAG CGA GGC TCC CGC ATC AGC TCC
578
Ile Asp Glu Met Asp Arg Arg Gln Glu Arg Gly Ser Arg Ile Ser Ser
15 20 25 30

GCC ATC GAC CGC GGC GAC ACC GAG ACG ACC ATG CCG TCC ATC AGC AGT
626
Ala Ile Asp Arg Gly Asp Thr Glu Thr Thr Met Pro Ser Ile Ser Ser
35 40 45

GAC CGC GCC GCC CTT TGT GGC GGC TGT GGC GGC AAG ATC TCG GAC CGC
674
Asp Arg Ala Ala Leu Cys Gly Gly Cys Gly Gly Lys Ile Ser Asp Arg
50 55 60

TAC TAC CTG CTG GCG GTG GAC AAG CAG TGG CAC ATG CGC TGC CTC AAG
722
Tyr Tyr Leu Leu Ala Val Asp Lys Gln Trp His Met Arg Cys Leu Lys
65 70 75

TGC TGC GAG TGC AAG CTC AAC CTG GAG TCG GAG CTC ACC TGT TTC AGC
770
Cys Cys Glu Cys Lys Leu Asn Leu Glu Ser Glu Leu Thr Cys Phe Ser
80 85 90

```
AAG GAC GGT AGC ATC TAC TGC AAG GAA GAC TAC TAC CGG CGC TTC TCT
818
Lys Asp Gly Ser Ile Tyr Cys Lys Glu Asp Tyr Tyr Arg Arg Phe Ser
 95                  100                 105                 110

GTG CAG CGC TGC GCC CGC TGC CAC CTG GGC ATC TCG GCC TCG GAG ATG
866
Val Gln Arg Cys Ala Arg Cys His Leu Gly Ile Ser Ala Ser Glu Met
                115                 120                 125

GTG ATG CGC GCT CGG GAC TTG GTT TAT CAC CTC AAC TGC TTC ACG TGC
914
Val Met Arg Ala Arg Asp Leu Val Tyr His Leu Asn Cys Phe Thr Cys
            130                 135                 140

ACC ACG TGT AAC AAG ATG CTG ACC ACG GGC GAC CAC TTC GGC ATG AAG
962
Thr Thr Cys Asn Lys Met Leu Thr Thr Gly Asp His Phe Gly Met Lys
        145                 150                 155

GAC AGC CTG GTC TAC TGC CGC TTG CAC TTC GAG GCG CTG CTG CAG GGC
1010
Asp Ser Leu Val Tyr Cys Arg Leu His Phe Glu Ala Leu Leu Gln Gly
    160                 165                 170

GAG TAC CCC GCA CAC TTC AAC CAT GCC GAC GTG CAG GCG GCG CGT GCA
1058
Glu Tyr Pro Ala His Phe Asn His Ala Asp Val Gln Ala Ala Arg Ala
175                 180                 185                 190

CGC GCG GCG GCC AAG AGC GCG GGG CTG GGC GCA GCA GGG GCC AAC CCT
1106
Arg Ala Ala Ala Lys Ser Ala Gly Leu Gly Ala Ala Gly Ala Asn Pro
                195                 200                 205

CTG GGT CTT CCC TAC TAC AAT GGC GTG GGC ACT GTG CAG AAG GGG CGG
1154
Leu Gly Leu Pro Tyr Tyr Asn Gly Val Gly Thr Val Gln Lys Gly Arg
            210                 215                 220

CCG AGG AAA CGT AAG AGT CCG GGC CCC GGT GCG GAT CTG GCG GCC TAC
1202
Pro Arg Lys Arg Lys Ser Pro Gly Pro Gly Ala Asp Leu Ala Ala Tyr
        225                 230                 235

ACA CGT GCG CTA AGC TGC AAC GAA AAC GAC GCA GAG CAC CTG GAC CGT
1250
Thr Arg Ala Leu Ser Cys Asn Glu Asn Asp Ala Glu His Leu Asp Arg
    240                 245                 250

GAC CAG CCA TAC CCC AGC AGC CAG AAG ACC AAG CGC ATG CGC ACG TCC
1298
Asp Gln Pro Tyr Pro Ser Ser Gln Lys Thr Lys Arg Met Arg Thr Ser
255                 260                 265                 270

TTC AAG CAC CAC CAG CTT CGG ACC ATG AAG TCT TAC TTT GCC ATT AAC
1346
Phe Lys His His Gln Leu Arg Thr Met Lys Ser Tyr Phe Ala Ile Asn
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                    275                 280                 285

CAC AAT CCC GAT GCC AAG GAC TTG AAG CAG CTC GCG CAA AAG ACG GGC
1394
His Asn Pro Asp Ala Lys Asp Leu Lys Gln Leu Ala Gln Lys Thr Gly
            290                 295                 300

CTC ACC AAG CGG GTC CTC CAG GTC TGG TTC CAG AAC GCC CGA GCC AAG
1442
Leu Thr Lys Arg Val Leu Gln Val Trp Phe Gln Asn Ala Arg Ala Lys
        305                 310                 315

TTC AGG CGC AAC CTC TTA CGG CAG GAA AAC ACG GGC GTG GAC AAG TCG
1490
Phe Arg Arg Asn Leu Leu Arg Gln Glu Asn Thr Gly Val Asp Lys Ser
    320                 325                 330

ACA GAT GCG GCG CTG CAG ACA GGG ACG CCA TCG GGC CCG GCC TCG GAG
1538
Thr Asp Ala Ala Leu Gln Thr Gly Thr Pro Ser Gly Pro Ala Ser Glu
335                 340                 345                 350

CTC TCC AAC GCC TCG CTC AGC CCC TCC AGC ACG CCC ACC ACC CTG ACA
1586
Leu Ser Asn Ala Ser Leu Ser Pro Ser Ser Thr Pro Thr Thr Leu Thr
                355                 360                 365

GAC TTG ACT AGC CCC ACC CTG CCA ACT GTG ACG TCC GTC TTA ACT TCT
1634
Asp Leu Thr Ser Pro Thr Leu Pro Thr Val Thr Ser Val Leu Thr Ser
            370                 375                 380

GTG CCT GGC AAC CTG GAG GCC ATG AGC CTC ACA GCC CCT CAC AAA CGA
1682
Val Pro Gly Asn Leu Glu Ala Met Ser Leu Thr Ala Pro His Lys Arg
        385                 390                 395

CTC TTA CCA ACC TTT TCT AAT GAC TCG CAA CCC CCT CAC CCC ACA ATT
1730
Leu Leu Pro Thr Phe Ser Asn Asp Ser Gln Pro Pro His Pro Thr Ile
    400                 405                 410

TCT TTA AAA AAG AAA TTA TCT TTA GTT TGAATTCCAA GTGTATTTTA
1777
Ser Leu Lys Lys Lys Leu Ser Leu Val
415                 420

AAATAGAGGC TTTGAGCAAC TAACTAACCA CATTTTAGGA TCTCGCCTGG AAACAGAGGT
1837

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1887
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 423 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Met Leu Phe His Ser Leu Ser Gly Pro Glu Val His Gly Val Ile Asp
 1               5                  10                  15

Glu Met Asp Arg Arg Gln Glu Arg Gly Ser Arg Ile Ser Ser Ala Ile
            20                  25                  30

Asp Arg Gly Asp Thr Glu Thr Thr Met Pro Ser Ile Ser Ser Asp Arg
        35                  40                  45

Ala Ala Leu Cys Gly Gly Cys Gly Gly Lys Ile Ser Asp Arg Tyr Tyr
    50                  55                  60

Leu Leu Ala Val Asp Lys Gln Trp His Met Arg Cys Leu Lys Cys Cys
65                  70                  75                  80

Glu Cys Lys Leu Asn Leu Glu Ser Glu Leu Thr Cys Phe Ser Lys Asp
                85                  90                  95

Gly Ser Ile Tyr Cys Lys Glu Asp Tyr Tyr Arg Arg Phe Ser Val Gln
            100                 105                 110

Arg Cys Ala Arg Cys His Leu Gly Ile Ser Ala Ser Glu Met Val Met
        115                 120                 125

Arg Ala Arg Asp Leu Val Tyr His Leu Asn Cys Phe Thr Cys Thr Thr
    130                 135                 140

Cys Asn Lys Met Leu Thr Thr Gly Asp His Phe Gly Met Lys Asp Ser
145                 150                 155                 160

Leu Val Tyr Cys Arg Leu His Phe Glu Ala Leu Leu Gln Gly Glu Tyr
                165                 170                 175

Pro Ala His Phe Asn His Ala Asp Val Gln Ala Ala Arg Ala Arg Ala
            180                 185                 190

Ala Ala Lys Ser Ala Gly Leu Gly Ala Ala Gly Ala Asn Pro Leu Gly
        195                 200                 205

Leu Pro Tyr Tyr Asn Gly Val Gly Thr Val Gln Lys Gly Arg Pro Arg
    210                 215                 220

Lys Arg Lys Ser Pro Gly Pro Gly Ala Asp Leu Ala Ala Tyr Thr Arg
225                 230                 235                 240

Ala Leu Ser Cys Asn Glu Asn Asp Ala Glu His Leu Asp Arg Asp Gln
                245                 250                 255

Pro Tyr Pro Ser Ser Gln Lys Thr Lys Arg Met Arg Thr Ser Phe Lys
            260                 265                 270
```

His His Gln Leu Arg Thr Met Lys Ser Tyr Phe Ala Ile Asn His Asn
275 280 285

Pro Asp Ala Lys Asp Leu Lys Gln Leu Ala Gln Lys Thr Gly Leu Thr
290 295 300

Lys Arg Val Leu Gln Val Trp Phe Gln Asn Ala Arg Ala Lys Phe Arg
305 310 315 320

Arg Asn Leu Leu Arg Gln Glu Asn Thr Gly Val Asp Lys Ser Thr Asp
325 330 335

Ala Ala Leu Gln Thr Gly Thr Pro Ser Gly Pro Ala Ser Glu Leu Ser
340 345 350

Asn Ala Ser Leu Ser Pro Ser Ser Thr Pro Thr Thr Leu Thr Asp Leu
355 360 365

Thr Ser Pro Thr Leu Pro Thr Val Thr Ser Val Leu Thr Ser Val Pro
370 375 380

Gly Asn Leu Glu Ala Met Ser Leu Thr Ala Pro His Lys Arg Leu Leu
385 390 395 400

Pro Thr Phe Ser Asn Asp Ser Gln Pro Pro His Pro Thr Ile Ser Leu
405 410 415

Lys Lys Lys Leu Ser Leu Val
420

What is claimed is:

1. A method for generating renewable stem cells capable of differentiating into hematopoietic lineages, which method comprises causing ectopic expression of a hematopoietic gene, the expression of which confers a self-renewable phenotype on the stem cell.

2. A method for generating renewable stem cells capable of differentiating into hematopoietic lineages, which method comprises transfecting a stem cell with a gene expression construct encoding a hematopoietic gene, and maintaining the cells under conditions wherein the hematopoietic gene is expressed at a level conferring a self-renewable phenotype of the transfected stem cell.

3. The method of claim 2, wherein the hematopoietic gene encodes a LIM-homeobox protein.

4. The method of claim 3, wherein the LIM-homeobox protein is *LH-2*.

5. The method of claim 4, wherein the *LH-2* protein is represented in any one of SEQ ID Nos. 2 or 4.

6. The method of any of claims 1-4, wherein the hematopoietic gene a gene involved in hematopoiesis characterized by a loss-of-function phenotype of a decrease in a relative number of hematopoietic cells.

7. The method of claim 2, wherein the stem cell is an embryonic stem cell.

8. The method of claim 3, wherein the stem cell is a hematopoietic stem cell.

9. The method of claim 8, wherein the hematopoietic stem cell is isolated from peripheral blood, cord blood or bone marrow.

10. The method of any of claims 2, 7 or 8, wherein the stem cell is isolated from a mammal.

11. The method of claim 10, wherein the mammal is a primate.

12. The method of claim 11, wherein the primate is a human.

13. The method of claim 10, wherein the mammal is a transgenic non-human mammal.

14. The method of claim 2, wherein the stem cell is transfected with the gene expression construct by viral-mediated gene transfer.

15. The method of claim 2, wherein the stem cell is transfected with the gene expression construct by liposome-mediated gene transfer.

16. The method of claim 2, wherein the stem cell is transfected with the gene expression construct *in vivo*.

17. The method of claim 2, wherein the stem cell is transfected with the gene expression construct *ex vivo*.

18. The method of claim 2, which method comprises the further step of expanding the transfected stem cell by contact with Steel factor.

19. The method of claim 2, which method comprises the further step of contacting the transfected stem cell with one or more factors selected from the group consisting of erythropoietin (EPO), thrombopoietin, granulocyte/macrophage colony stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), and an interleukin.

20. A substantially pure population of viable stem cells, which cells have been genetically modified to ectopically express a hematopoietic gene, the expression of which confers a self-renewable phenotype on the stem cell.

21. The cells of claim 20, wherein the hematopoietic gene encodes a LIM-homeobox protein.

22. The cells of claim 21, wherein the LIM-homeobox protein is *LH-2*.

23. The cells of claim 22, wherein the *LH-2* protein is encoded by a gene which hybridizes under stringent conditions to the nucleic acid represented in any one of SEQ ID Nos. 1 or 3.

24. The cells of any of claims 20-23, wherein the hematopoietic gene a gene involved in hematopoiesis characterized by a loss-of-function phenotype of a decrease in a relative number of hematopoietic cells.

25. The cells of claim 20, wherein the stem cell is an embryonic stem cell.

26. The cells of claim 20, wherein the stem cell is a hematopoietic stem cell.

27. The cells of claim 26, wherein the hematopoietic stem cell is isolated from peripheral blood, cord blood, fetal liver or bone marrow.

28. The cells of claim 20, wherein the stem cell is isolated from a mammal.

29. The cells of claim 28, wherein the mammal is a primate.

30. The cells of claim 29, wherein the primate is a human.

31. The cells of claim 28, wherein the mammal is a transgenic non-human mammal.

32. A purified preparation of cells comprising differentiated hematopoietic cells of the stem cells of claim 20.

33. The cells of claim 32, which differentiated hematopoietic cells stem cells are selected from the group consisting of myeloid lineages, lymphoid lineages, erythroid colonies, and combinations thereof.

34. A pharmaceutical composition comprising the cellular composition of any of claims 20 or 32.

35. A method for treating a disorder characterized by a reduced number of hematopoietic precursors or their progeny, in a subject animal, comprising introducing into the subject the pharmaceutical composition of claim 34.

36. The method of claim 35, wherein the disorder results from a bone marrow deficiency.

37. The method of claim 35, wherein the disorder results from a cancer treatment involving radio- or chemotherapy.

38. The method of claim 35, wherein the disorder is an inherent or acquired anemia.

39. A method for treating a disorder characterized by an alteration in the immune system, in a subject, comprising introducing into the subject the pharmaceutical composition of claim 34.

40. The method of claim 39, wherein the disorder is an auto-immune disease.

41. The method of claim 39, wherein the disorder is AIDS.

42. A stem cell which has been genetically modified to ectopically express a hematopoietic gene, the expression of which confers a self-renewable phenotype on the stem cell.

43. A conditioned media of generated by a stem cell culture comprising stem cells which ectopically express a hematopoietic gene which confers a self-renewable phenotype on the stem cell.

44. A purified or semi-purified preparation of one or more factors generated by a stem cell culture comprising stem cells which ectopically express a hematopoietic gene which confers a self-renewable phenotype on the stem cell.

45. The conditioned media of claim 43 or the preparation of claim 44, which hematopoietic gene encodes a LIM-homeobox protein.

46. A method for isolating a factor which confers a self-renewable phenotype on a hematopoietic stem cell comprising (i) providing a stem cell culture including modified stem cells that ectopically express a hematopoietic gene which confers a self-renewable phenotype on the stem cells; and (ii) purifying to some degree one or more factors generated by the stem cell culture, which factors induce a self-renewable phenotype when applied to an unmodifed hematopoietic stem cell.

47. A method for obtaining a coding sequence for a factor which confers a self-renewable phenotype on a hematopoietic stem cell comprising (i) contacting a stem cell culture with the purified factor(s) of claim 46; and (ii) cloning one or more genes expressed in the stem cells which encode factor(s) generated by the stem cell culture,

which factor(s) induce a self-renewable phenotype when applied to an unmodifed hematopoietic stem cell.

48. A method for obtaining a coding sequence for a factor which confers a self-renewable phenotype on a hematopoietic stem cell comprising (i) providing a stem cell culture including modified stem cells that ectopically express a hematopoietic gene which confers a self-renewable phenotype on the stem cells; and (ii) cloning one or more genes expressed in the modified stem cells which encode factor(s) generated by the stem cell culture, which factor(s) induce a self-renewable phenotype when applied to an unmodifed hematopoietic stem cell.

49. The method of claim 47 or 48, wherein the cloned gene(s), or a nucleic acid encoding an identical or homologous protein, is provided in an expression vector for recombinantly expressin the factor(s).

50. The method of claim 46, 47 or 48, wherein the factor(s) is formulated in a pharmaceutically acceptable carrier.

51. A purified or semi-purified preparation of one or more factors isolated according to any of claims 46-50.

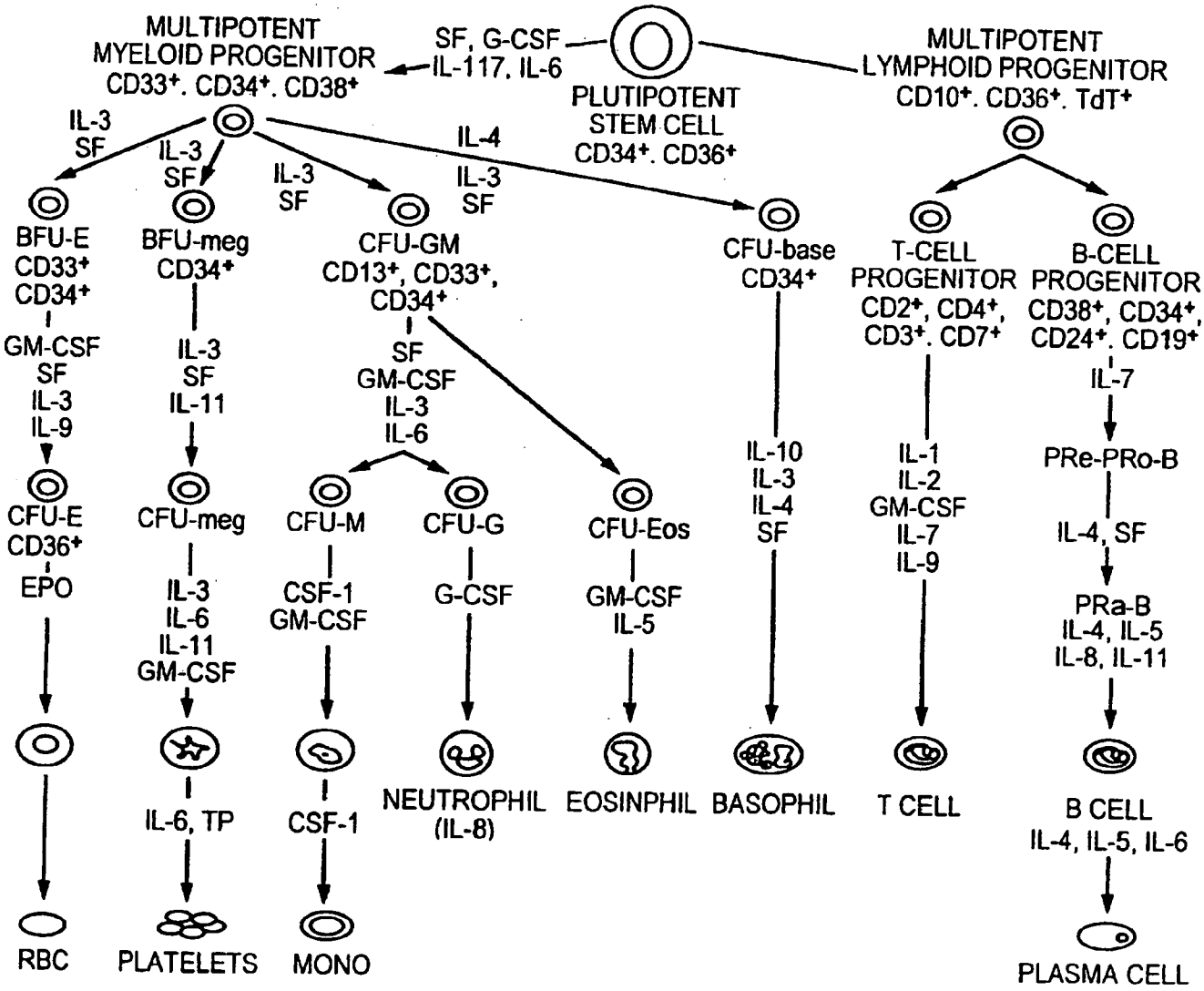

MULTIPOTENT
MYELOID PROGENITOR
CD33+. CD34+. CD38+
SF, G-CSF
IL-117. IL-6
PLUTIPOTENT
STEM CELL
CD34+. CD36+
MULTIPOTENT
LYMPHOID PROGENITOR
CD10+. CD36+. TdT+
IL-3
SF
IL-3
SF
IL-3
SF
IL-4
IL-3
SF
BFU-E
CD33+
CD34+
BFU-meg
CD34+
CFU-GM
CD13+, CD33+,
CD34+
CFU-base
CD34+
T-CELL
PROGENITOR
CD2+, CD4+,
CD3+. CD7+
B-CELL
PROGENITOR
CD38+, CD34+,
CD24+. CD19+
GM-CSF
SF
IL-3
IL-9
IL-3
SF
IL-11
SF
GM-CSF
IL-3
IL-6
IL-10
IL-3
IL-4
SF
IL-1
IL-2
GM-CSF
IL-7
IL-9
IL-7
PRe-PRo-B
IL-4, SF
PRa-B
IL-4, IL-5
IL-8, IL-11
CFU-E
CD36+
EPO
CFU-meg
CFU-M
CFU-G
CFU-Eos
IL-3
IL-6
IL-11
GM-CSF
CSF-1
GM-CSF
G-CSF
GM-CSF
IL-5
IL-6, TP
CSF-1
NEUTROPHIL
(IL-8)
EOSINPHIL
BASOPHIL
T CELL
B CELL
IL-4, IL-5, IL-6
RBC
PLATELETS
MONO
PLASMA CELL

FIG. 1

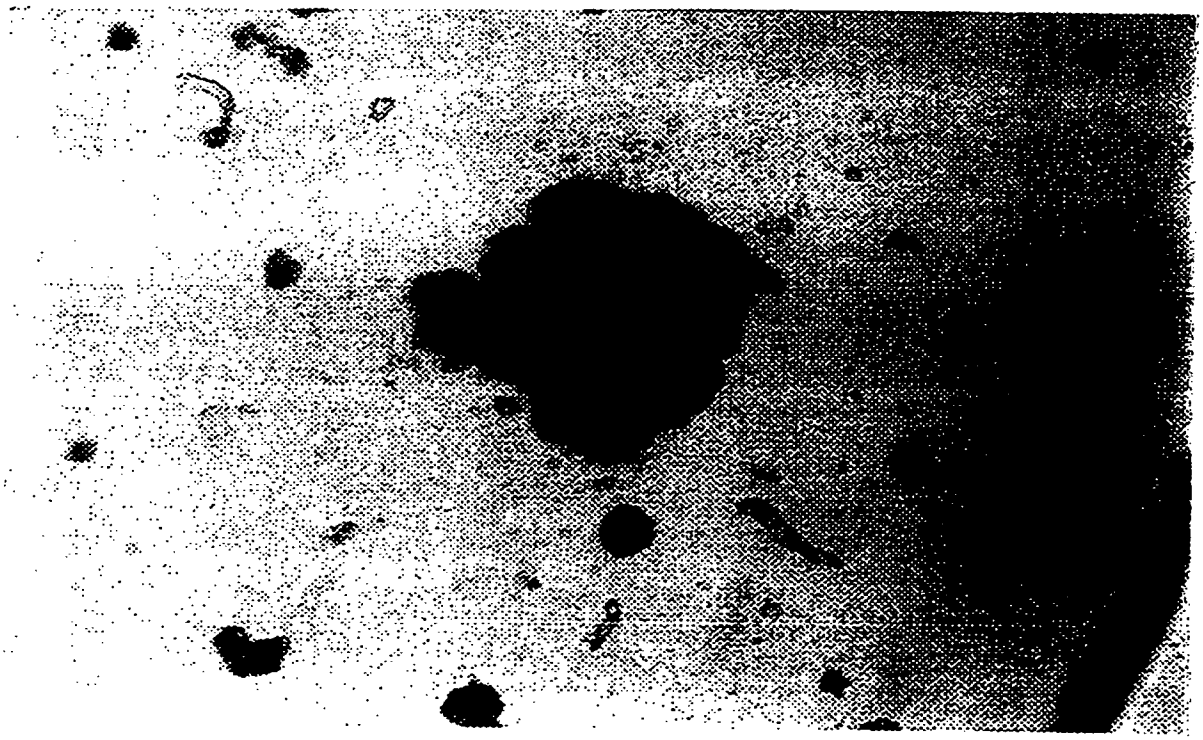


FIG. 2

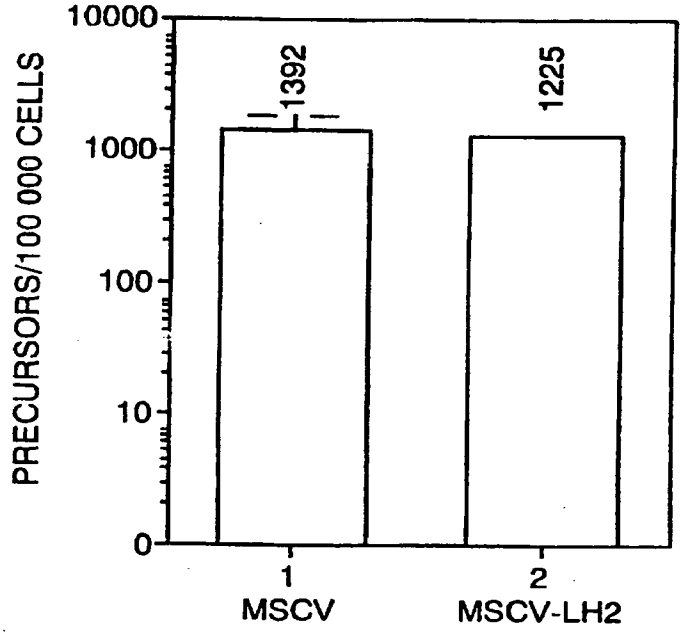

10000
1000
100
10
0
PRECURSORS/100 000 CELLS
1392
1225
1
MSCV
2
MSCV-LH2

FIG. 3A

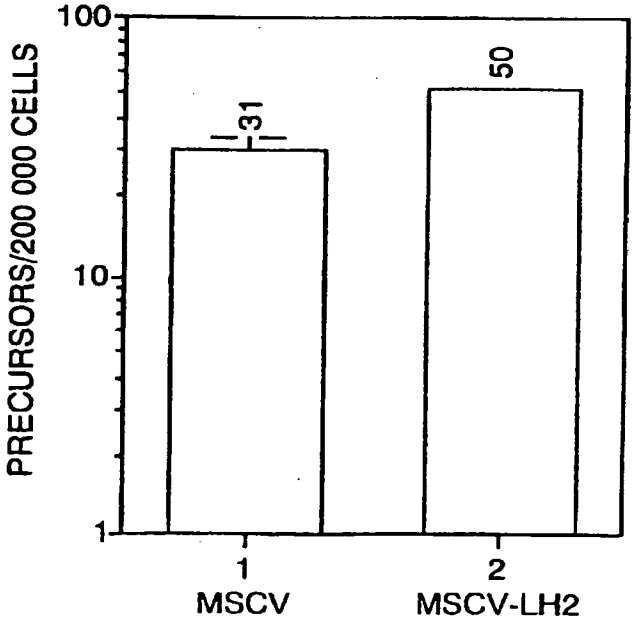

100
10
1
PRECURSORS/200 000 CELLS
31
50
1
MSCV
2
MSCV-LH2

FIG. 3B

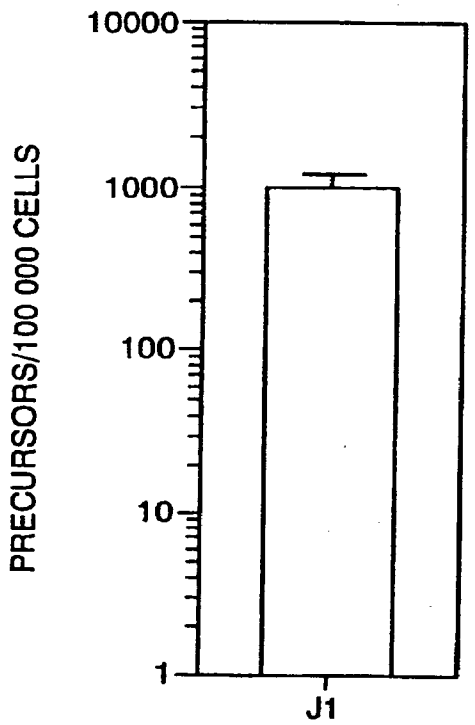
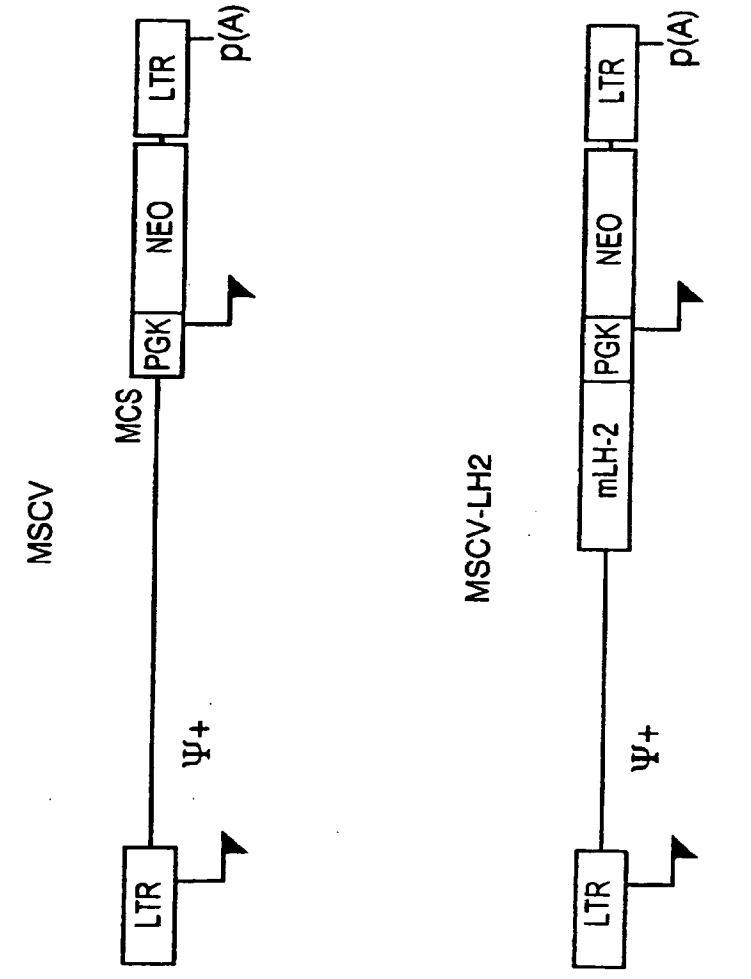


FIG. 4

FIG. 5



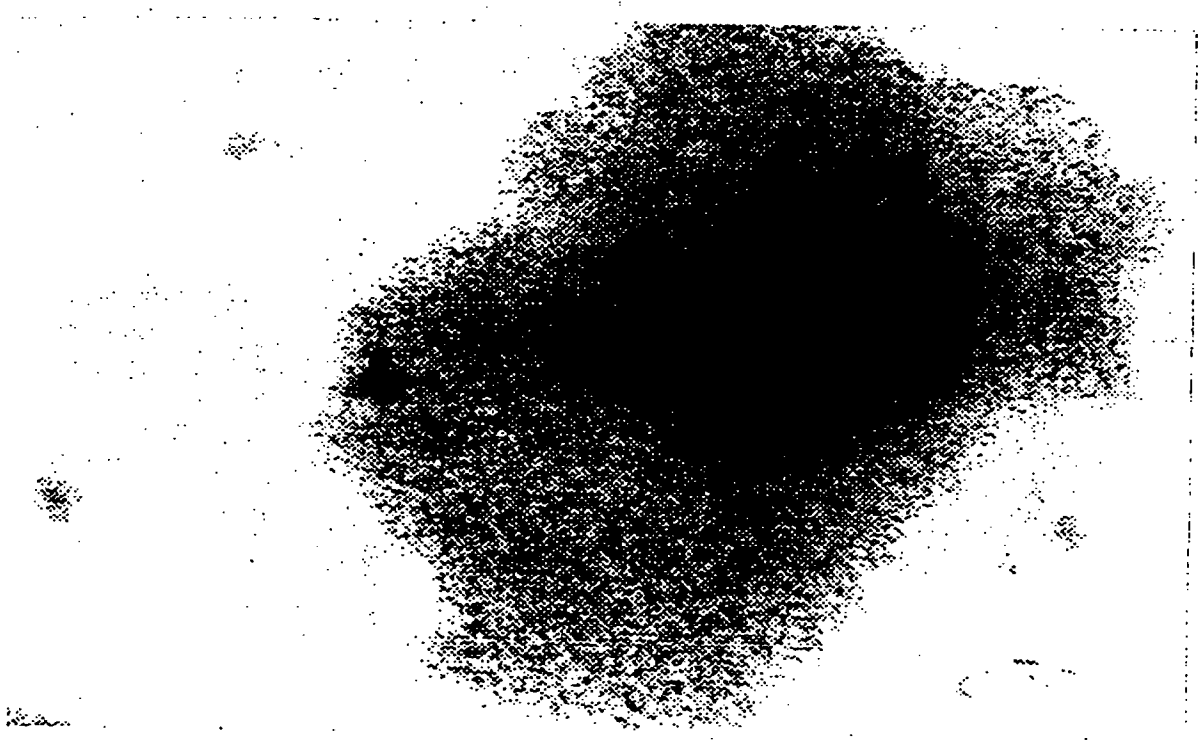


FIG. 6

Figure 7

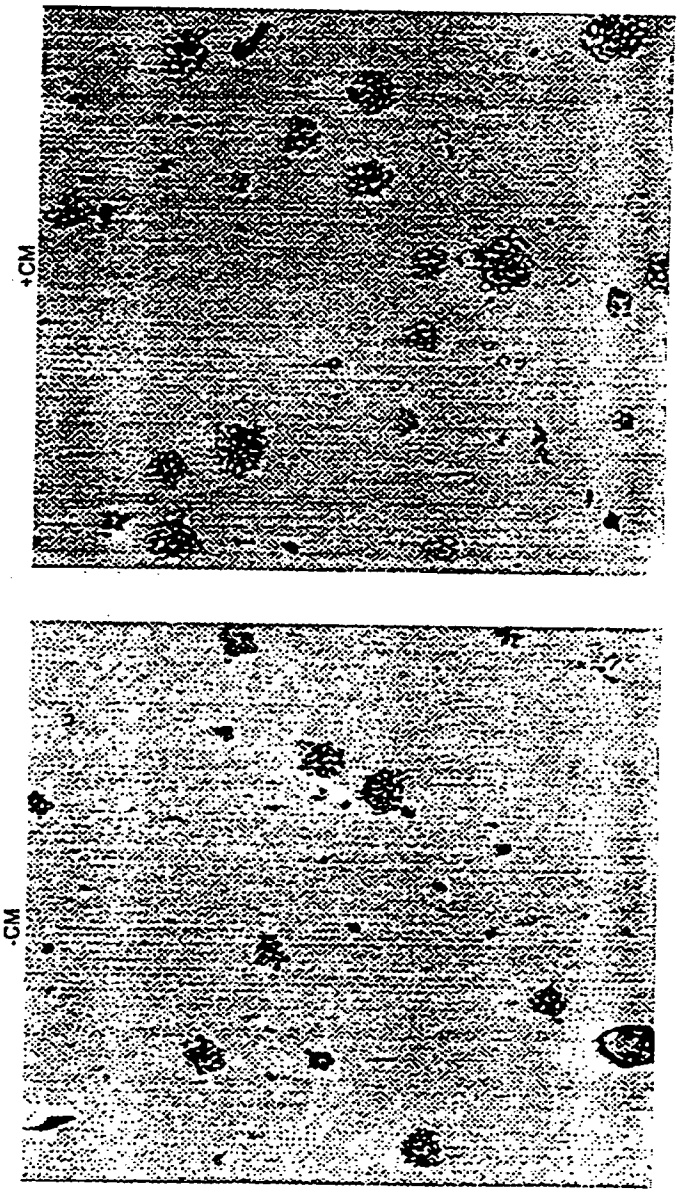
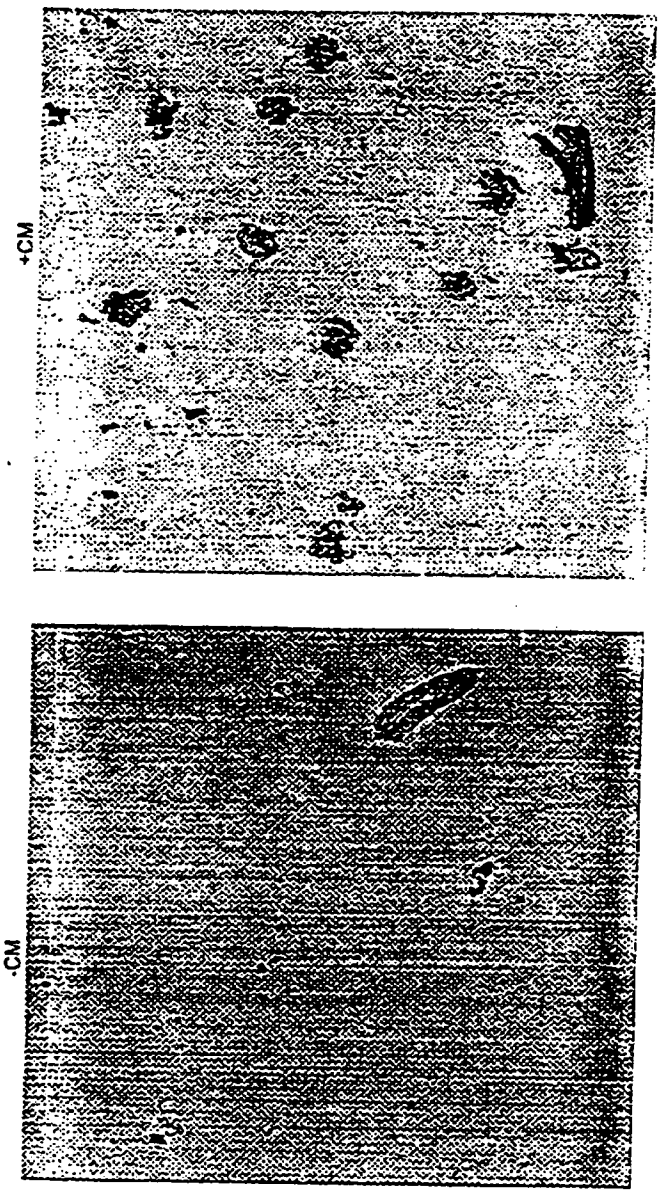


Figure 8



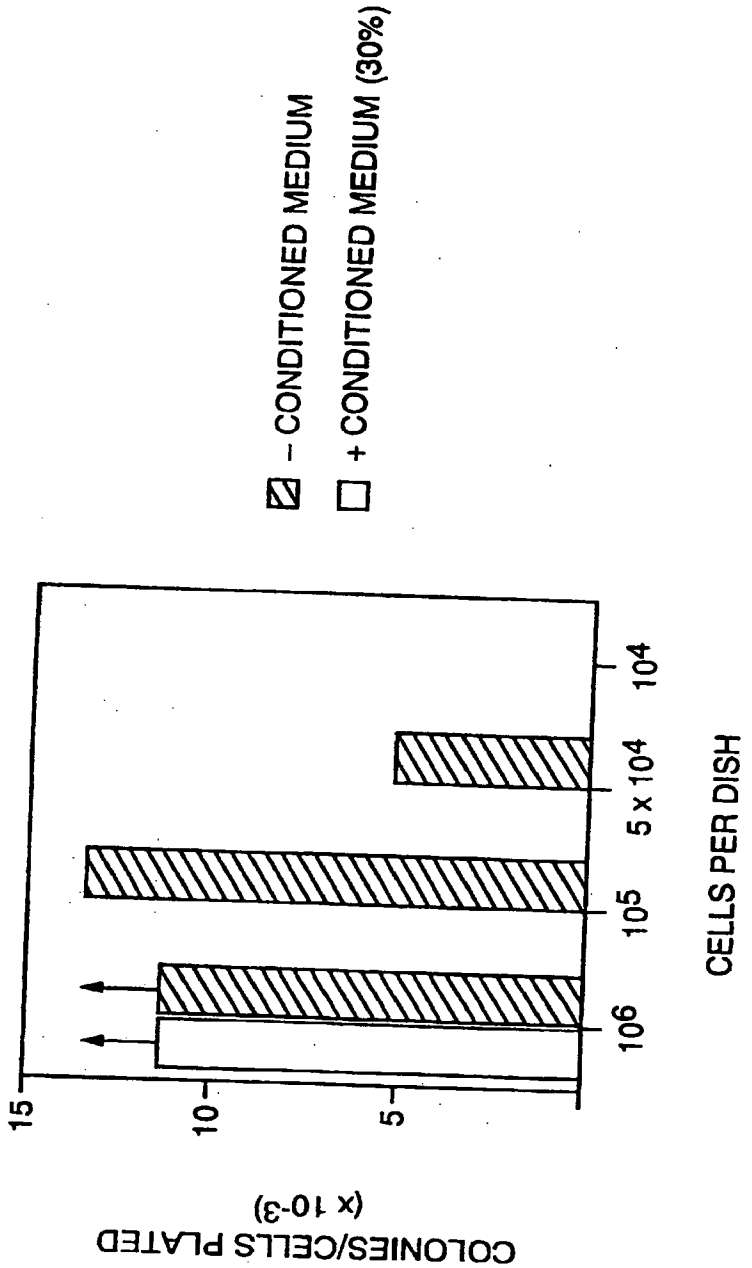

– CONDITIONED MEDIUM
+ CONDITIONED MEDIUM (30%)
15
10
5
(x 10-3)
COLONIES/CELLS PLATED
10⁶
10⁵
5 x 10⁴
10⁴
CELLS PER DISH

FIG. 9

INTERNATIONAL SEARCH REPORT

In. ..ational Application No
PCT/IB 97/01246

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/08 C12N5/10 A61K48/00 C07K14/47 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PEROOTI D. ET AL: "Overexpression of the zinc finger protein MZF1 inhibits hematopoietic development from embryonic stem cells: correlation with negative regulation of CD34 and c-myb promoter activity" MOLECULAR AND CELLULAR BIOLOGY, vol. 15, no. 11, 1995, pages 6075-6087, XP002053235 see the whole document --- -/--	1,2, 6-20, 24-44, 46-51

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 January 1998	17.02.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fernandez y Branas, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In. .ational Application No
PCT/IB 97/01246

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DEGUCHI, Y. ET AL: "A diverged homeobox gene is involved in the proliferation and lineage commitment of human hematopoietic progenitors and highly expressed in acute myelogenous leukemia" BLOOD, vol. 79, no. 11, 1992, pages 2841-2848, XP002053236 see the whole document	1,2, 6-20, 24-44, 46-51
A	YANG XU ET AL: "LH-2: a LIM/homeodomain gene expressed in developing lymphocytes and neural cells" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, 1993, WASHINGTON US, pages 227-231, XP002053237 cited in the application see the whole document	1-51
A	PEDERSEN R.A. ET AL: "Studies of in vitro differentiation with embryonic stem cells" REPROD. FERTIL. DEV., vol. 6, 1994, pages 543-552, XP002053238 see the whole document	1-51
A	WO 96 09400 A (SYSTEMIX INC ;UNIV LELAND STANFORD JUNIOR (US)) 28 March 1996 cited in the application see the whole document	1-51

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 97/01246

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/IB 97/01246

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 1-16, 18-19, 35-41
are directed to a method of
treatment of the human/animal body , the search has been carried out and
based on the alleged effects of the composition.

INTERNATIONAL SEARCH REPORT

Int. ational Application No
PCT/IB 97/01246

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9609400 A	28-03-96	AU 3635695 A	09-04-96

Form PCT/ISA/210 (patent family annex) (July 1992)